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ON THE MECHANISM OF IMMUNE-ADHERENCE

I. Differentiation from Acid-Adhesion of Bacteria to Erythrocytes

INTRODUCTION

Immune-adherence (IA) has been described as a specific immunological reaction wherein microorganisms or other particulate antigens sensitized with antibody (Ab) and complement (C′) become attached to the surface of human or monkey erythrocytes.[20, 25] Certain practical advantages of IA noted in recent studies have emphasized the necessity for an understanding of the fundamental nature of the reaction. For example, the marked sensitivity of IA permits the detection of Ab concentrations as low as .01 to .005 μg. Ab nitrogen (N) per ml. of serum[30]; a wide range of bacteria,[15–18, 28] viruses,[20, 26] and soluble antigens,[28] react in IA; and some applications of IA as a diagnostic test for syphilis[5, 6, 23] have been reported. The technique for measuring IA has been simplified considerably by the development of a hemagglutination assay.[30] Also, there is evidence that the attachment of bacteria to erythrocytes enhances phagocytosis of the microbes *in vitro*. This latter observation, coupled with the demonstration that IA occurs *in vivo*, has suggested a possible role of erythrocytes in defense of the host against infectious agents.[26] A final feature of some practical importance concerns the observation that C′ in human serum is as active as guinea-pig C′, e.g., 1 ml. of normal human serum diluted to 1/400 ± 10 per cent provides adequate C′ for IA with a variety of antigens. Reproducible reactivity at such high serum dilutions provides a latitude for measurement of decline in disease states which often was difficult to detect by the classical hemolytic assay using sheep erythrocytes and rabbit antiserum wherein C′ reactivity was lost at dilutions of human serum exceeding 1/40 or 1/60. The use of IA in studies of human C′ has demonstrated a definitive requirement for both Ca^{++} and Mg^{++}.[41]

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These observations have stimulated our efforts to comprehend the quantitative aspects of IA and in particular the nature of the bond established between the sensitized particulate antigen and the normal erythrocyte. The latter has proved elusive to date but during the course of our investigation conditions have been defined under which two different reactions occur which mimic IA in the final result. These two phenomena tentatively have been termed "acid-adhesion" and "C′-dependent mixed aggregation" and are described in this and the following paper, respectively. With attention to detail, it is simple to differentiate these three reactions, all of which are influenced by both Ab and C′. The importance of such differentiation is emphasized by the recent report of Lamanna and Hollander[20] which casts misleading doubts on the specificity of the primate erythrocyte in IA. Further, an extensive review of the literature on "serological adhesion" by Lamanna[19] demonstrates the confused and often contradictory information which exists in this potentially useful immunological area.

MATERIALS AND METHODS

Bacteria. Various organisms were harvested from broth cultures after 6 to 8 hours' incubation at 37° C. The bacteria were killed either by heat (65° C. for 20 min.) or by formalin added to a final concentration of 0.5 per cent, or by both methods. They were washed twice in sterile saline which had been boiled to remove dissolved CO_2 or in saline containing 0.5 per cent formalin, and stored as a concentrated suspension in the same fluid in a refrigerator at 0-4° C. (hereafter referred to as 2° C.). Assays for IA and for acid-adhesion gave quantitatively similar results with washed living cultures and with heat- or formalin-killed cultures.

(a) *Diplococcus pneumoniae,* Types III, VIII, XIII, and XXXI (hereafter referred to as Pn III, Pn VIII, etc.) were passed through mice and grown in brain heart infusion broth containing 0.5 per cent glucose, 0.1 per cent $NaHCO_3$, and 20 mg. per cent glutamine.

(b) *Escherichia coli,* strain K12, and *Micrococcus pyogenes,* var. albus, were grown in beef heart infusion broth supplemented with .002 per cent para-amino-benzoic acid.

(c) *Salmonella typhosa,* strain 0901, was grown on Difco nutrient agar in Blake bottles. The bacteria were washed off the surface of the agar with sterile saline and the resulting suspension killed, washed, and resuspended in sterile saline.

Rickettsia. R. burneti (egg-adapted, American 9 Mile strain) generously was supplied by Dr. Herald Cox of Lederle Laboratories. This concentrated suspension was diluted 1/200 and washed in SAVB++ by high speed centrifugation.

Spirochetes. Four batches of *Treponema pallidum* were isolated from testicular syphilomata of rabbits at a maximum of 8 days after inoculation. Three batches were from rabbits which had been exposed to 600 r total body X-irradiation before inoculation. X-ray treatment was given to inhibit Ab formation and so avoid the risk of isolating organisms already sensitized with Ab *in vivo.* These treponemal suspensions were generously supplied by Sylvana Chemical Co., Orange, New Jersey.

Starch granules. Suspensions of washed granules of rice starch (Fisher) and of *Amaranthus cruentus* starch (U. S. Department of Agriculture, Peoria, Illinois) were prepared at a concentration of 20 mg. per ml. in sterile saline and stored at 2° C. These varieties were selected because of their small and uniform sizes (average diameters 5 mμ. and 1 mμ. respectively) and because it has been postulated that Ab in normal serum will sensitize the granules so that they react in a variety of immunological phenomena.[7, 29, 33]

Antibody. (a) Antisera to Pn XIII, Pn XXXI, and *S. typhosa* were prepared by injection of formalinized intact bacteria into rabbits. Pooled sera were heated at 56° C. for 60 min. and stored at —38° C. The Ab N contents of these sera were determined by micro-Kjeldahl analysis of the precipitates from mixtures of the soluble polysaccharides with the anti-pneumococcal sera and of the agglutinated bacteria from mixtures of *S. typhosa* with its antiserum.

(b) Human syphilitic sera were obtained through the courtesy of Dr. C. R. Rein. These were from patients with proved syphilis and had been found reactive in the treponemal immobilization test.

(c) In several instances sera from adult normal individuals were used as a source of "natural" Ab. Because heating often resulted in a decrease of Ab activity, these sera could not be heated to provide antibody free from C′. Therefore, when indicated, the particulate antigen was treated with fresh serum for a short time, usually 30 min., at exactly 0° C. in the presence of 0.01 *M* EDTA (trisodium ethylene diamine tetra-acetate) and then washed in buffer before assay. We have been unable to demonstrate any significant uptake of C′ on the resultant antigen-antibody complex under these circumstances.

Throughout this work it has been necessary to avoid marked agglutination of bacteria. This was usually done by using dilute suspensions of antigens and relatively high dilutions of antisera. The IA_{50} dose of Ab was determined as the amount of antiserum required to induce specific adherence of 50 per cent of a standardized suspension of bacteria to human E in the presence of "excess" C′ as outlined below. For optimal sensitization, 3 IA_{50} doses of Ab were used. With bacteria this usually represented a one plus or a trace agglutinating dose.

Complement. Pooled normal guinea pig serum (Carworth Farms) was used as a source of C′. The hemolytic titer was between 200 and 210 $C'H_{50}$ units per ml. Normal human sera also were used as C′, as indicated in specific protocols. Sera used as sources of C′ were stored in an electric refrigerator at —38° C. and handled at 0° C. after thawing.

The $C'IA_{50}$ titer was determined as that amount of serum which induced specific adherence of 50 per cent of a standardized suspension of bacteria or starch granules to human red cells when the antigens were sensitized with about 3 IA_{50} doses of Ab. Under these circumstances both human and guinea pig were ordinarily reactive at dilutions of approximately 1/400 and 1/300, respectively. In assays in which "excess" C′ was desired for sensitization of the antigen-Ab complex, we generally employed 1 ml. of a 1/20 or 1/40 dilution of serum or its equvialent, i.e., 10 to 20 $C'IA_{50}$ doses.

Erythrocytes (E). Human, sheep, rabbit, guinea pig, and chicken cells were used. Blood was drawn aseptically from either a vein (human, sheep, chicken) or the heart (rabbit, guinea pig) and stored in modified Alsever's solution at 2° C. Prolonged storage (about one month) did not influence the reactivity of the cells in either IA or

acid-adhesion. Before use, the cells were washed three times and made up to a 2 per cent suspension either in saline or in an appropriate buffer; the buffy coat was removed after each centrifugation. For IA the suspension was adjusted so that exactly 1 ml. diluted to 10 ml. with water gave an optical density (O.D.) of .395 when examined at a wave length of 541 on the Beckman DU spectrophotometer. In experiments on acid-adhesion, chicken cells were used most frequently, since they were less subject to gross distortion at low pH than were the E of other species.

Buffers. A stock of 5 times isotonic veronal buffer was prepared by dissolving 4.6 gm. 5,5 diethyl barbituric acid in 500 ml. of hot distilled water and then adding this solution to a second solution containing 83.8 gm. sodium chloride, 2.52 gm. sodium bicarbonate, and 3 gm. 5,5 diethyl barbital. After cooling to room temperature, the total volume was adjusted to 2,000 ml. with distilled water. For sensitization of antigens and for IA, isotonic veronal buffer (pH 7.3 to 7.6) containing 0.0005 *M* Mg++, 0.00015 *M* Ca++, and 0.1 per cent bovine serum albumin was used (SAVB++). In some instances, the albumin was omitted (VB++). To cover a wide range of pH values a glycine-acetate-phosphate buffer series was used.[81]

Sensitization of organisms. The stock suspension of bacteria or starch granules was centrifuged, washed, and resuspended in SAVB++ to a suitable concentration. Dilutions of Ab and of C′ were prepared in SAVB++. Equal volumes of organisms, Ab, and C′ were incubated at 37° C. for 60 min. If the volume of the reaction mixture was less than 1 ml., this was done in a water bath with periodic shaking by hand. Larger volumes were incubated in a 37°-oven with continuous mechanical rotation (about 6 rpm). After incubation the mixtures were diluted in ice-cold saline, centrifuged at 0° C., washed, and resuspended to their original concentration in either SAVB++ (for IA) or saline (for acid-adhesion). The washing removed traces of serum proteins which might interfere with the buffer system at low pH. The removal of natural hemagglutinins from guinea pig serum was essential when IA assays were made by the hemagglutination pattern technique.[80] With nearly all the bacteria some slight to moderate clumping occurred as a result of sensitization with Ab and rapid centrifugation.

Reaction of antigens and indicator particles. (a) Immune-adherence: 0.5 ml. of SAVB++, 0.1 ml. of spectrophotometrically standardized human E, and 0.1 ml. of washed sensitized particulate antigen were incubated at 37° C. for 30 to 40 min. Unsensitized particles and particles sensitized with Ab only were included as controls in all cases. The mixtures were shaken periodically in the first 10 min., and then the erythrocytes were permitted to settle at 37° C. Adherence was detected either by the production of a hemagglutination pattern, or by counting the percentage of particles adherent to E by means of darkfield microscopic examination. At least 50 organisms were counted.

(b) Acid-adhesion: 0.5 ml. of glycine-acetate buffer and 0.1 ml. of a suspension of the indicator particle were incubated at 37° C. for 10 to 20 min. Erythrocytes and platelets of some species agglutinated at low pH and if antigens were added immediately, nonspecific trapping in these clumps occurred. It was also found that the change in red cells, induced by acid, took an appreciable time to occur and was most rapid at 37° C. After this pre-incubation 0.1 ml. of antigen was added and the mixture incubated at 37° C. or at room temperature for 30 min. The percentage of antigen particles adherent was determined by darkfield microscopic examination. In all experiments in which the

percentage of antigen particles adherent was counted, erythrocytes or other indicator particles were present in excess.

Precision of counting. When duplicate counts of the percentage of adherent particles were made on several samples of the same reaction mixture by two observers, the error was found to be ± 6 per cent.

Alsever's solution (modified). Distilled water was added to 2.05 gm. glucose, 0.8 gm. trisodium citrate, and 0.42 gm. sodium chloride, to make a total volume of 100 ml. The pH of the solution was adjusted to 6.1 with 5 per cent citric acid.

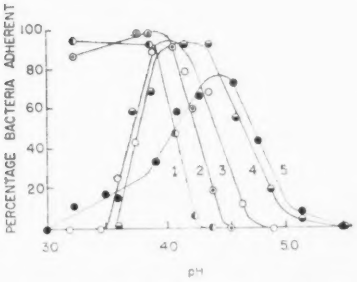

FIG. 1. The percentage of bacteria attached to chicken erythrocytes at varying pH.

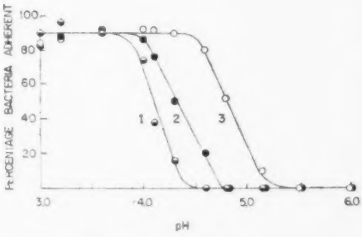

FIG. 2. The adhesion of *D. pneumoniae*, type XXXI to chicken erythrocytes at varying pH.

Curve	*Bacteria*
1	Pneumococcus, type XIII
2	Pneumococcus, type VIII
3	Pneumococcus, type III
4	*M. albus*
5	*E. coli*

Curve	*Treatment of pneumococci*
1	None
2	Antibody only
3	Antibody and complement

EXPERIMENTAL RESULTS

A. ATTACHMENT OF BACTERIA TO INDICATOR PARTICLES AT LOW pH

1. *Adhesion of unsensitized antigens to chicken E at low pH.* Saline suspensions of unsensitized antigens were incubated with chicken red cells as described above. The following bacteria were used: Pn III, Pn VIII, Pn XIII, Pn XXXI, *E. coli, M. albus,* and *S. typhosa.*

The percentage of organisms adherent is plotted against pH in Figure 1. Pn XXXI showed essentially the same type curve as Pn XIII and is not plotted since its characteristics are equally well shown in Figure 2. *S. typhosa* did not adhere over the pH range tested. No adherence was seen at any time with any bacteria above pH 5.5. Higher pH values are therefore omitted from all graphs. It is clear that the curves differ markedly from one

another and that for each of the bacteria tested the curve was fairly characteristic. With all the bacteria maximum adherence occurred at or below pH 4.5. The behavior below this point varied.

Below pH 5.5 certain changes were seen in the red cells. These were: (a) lysis, (b) spontaneous agglutination, and (c) a change in the color of the suspension from red to brown, probably indicating the formation of acid hematin.

2. *Adhesion of sensitized antigens to chicken red cells at low pH.* Exper. 041657: Washed suspensions of Pn XXXI (unsensitized, sensitized with rabbit Ab, and sensitized with both Ab and guinea pig C′) were incubated with chicken E at various pH's (Fig. 2). Sensitization with Ab alone shifted the curve significantly to the right, i.e., adhesion occurred at a higher pH than when unsensitized organisms were used. This effect was more marked when the organisms were sensitized with both Ab and C′. Similar results were obtained with Pn XIII. A number of other bacteria were tested, as well as *A. cruentus* starch. All were sensitized with sufficient Ab and C′ to allow about 70 per cent of the particles to react in IA with human E. With the exception of *E. coli,* for which normal rabbit serum provided the Ab, and *A. cruentus* starch, for which normal human serum was used as Ab, immune rabbit sera were used. Guinea pig C′ was used in all cases. The data on adhesion at various pH levels are collected from several separate assays and plotted in Figure 3.

When these curves are compared, a remarkable degree of uniformity is apparent as regards (a) the pH above which adhesion decreases markedly (4.5 in all cases) and (b) the pH above this at which adhesion is approximately half maximal (4.8 in all cases). This is in sharp contrast with the widely differing behavior of the various antigens when unsensitized (Fig. 1). Similar uniformity was not apparent below pH 4.5, where the behavior tended to return to that of the unsensitized antigen. Nor were similar results obtained with organisms sensitized with Abalone: Pn XIII and Pn XXXI behaved similarly when sensitized with Ab, but neither *S. typhosa* nor *A. cruentus* starch adhered unless exposed to C′ as well. *E. coli* behaved almost identically no matter what the extent of sensitization, the adhesion of Ab-C′ sensitized organisms at pH's above 4.5 being only slightly greater than that of unsensitized, while treatment with Ab alone did not change the curve at all.

The behavior of all the Ab-C′ sensitized antigens suggests that sensitization with Ab and C′ makes their surfaces similar from the point of view of electrostatic charge, at least at pH of 4.5 and above. If this be so, then the differences in behavior below pH 4.5 might be due to dissociation of Ab and

C′ from the antigen. The irregular results with Ab-sensitized antigens could well be due to dissociation, as Singer and Campbell[85] have evidence that other antigen-Ab precipitates dissociate below pH 4.5. The original surface could thus be uncovered resulting in a reactivity similar to that of the unsensitized particle. In all instances, however, it should be noted that Ab and C′ failed to induce attachment at pH 6.5 to 7.5, thus supporting our original contention that IA does not occur with nonprimate erythrocytes.

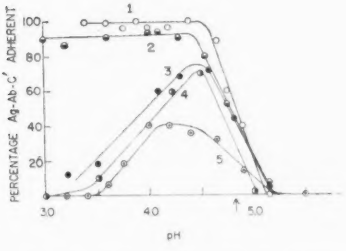

FIG. 3. The attachment to chicken erythrocytes of bacteria pretreated with antibody and guinea pig complement.

Curve	*Bacteria*
1	*D. pneumoniae*, type XIII
2	*D. pneumoniae*, type XXXI
3	*E. coli*
4	*A. cruentus* starch
5	*S. typhosa*

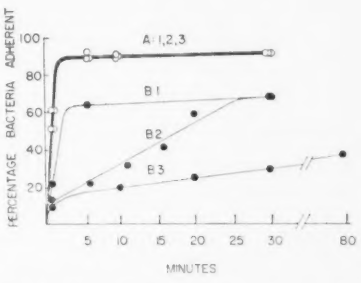

FIG. 4. The rate of attachment of pneumococci to erythrocytes via immune-adherence (series B) and acid-adhesion (series A).

Temperature	*Curves*	
37° C.	A1	B1
24°	A2	B2
0°	A3	B3

3. *Adhesion to erythrocytes of other species.* A series of experiments similar to those with chicken E was performed using Pn XIII mixed with rabbit, sheep, or guinea pig E as the indicator particle. Similar though not identical results were obtained. The red cells underwent the same changes as did those of the chicken, except that the ghosts formed by lysis at low pH were much more distorted, making microscopic counts more difficult. Rabbit and guinea pig cells behaved similarly to chicken cells, but the highest pH values for 100 per cent and 50 per cent adherence to sheep cells were slightly lower than the corresponding values for the cells of other species.

The adhesion of unsensitized or Ab-sensitized Pn XIII to human E also was similar to that seen with red cells of chicken, guinea pig, and rabbit. Ab-C′ sensitized organisms adhered to human E above pH 5.5, presumably due to true IA. However, at lower pH's the curve followed that of acid-

adhesion and it was not possible to separate the two phenomena. When sensitized *A. cruentus* starch was used, the degree of adherence to human erythrocytes was approximately constant from pH 4 to pH 9.

In another series of experiments red cells were lysed in distilled water before exposure to low pH or before reaction with sensitized antigens in IA. The curves of acid-adhesion were found to be shifted to the right. For example, 50 per cent of Ab-C′ sensitized Pn XIII were adherent to intact guinea pig red cells at pH 4.6, while the same percentage was adherent to lysed guinea pig cells at pH 4.9. With sensitized Pn XXXI and chicken E the corresponding figures were 4.8 and 5.0. Lysed human cells were less reactive in IA than were intact cells.

4. *Adhesion to particles other than erythrocytes.* (a) Immune-adherence: Other blood and tissue cells have been assayed from time to time for their reactivity in IA. *T. pallidum* sensitized with human Ab and C′, washed sensitized Pn XIII, and washed sensitized rice starch granules all gave negative results with human platelets at neutral pH. Monkey kidney cells from tissue cultures did not react in IA with either sensitized Pn XIII or with sensitized rice starch. Negative results were obtained when *E. coli* was used as the indicator particle in IA with a variety of washed sensitized antigens.

(b) Acid-adhesion: At low pH, monkey kidney cells, human platelets, and *E. coli* were all capable of combining with Pn XIII and with rice starch granules. For example, 44 per cent of the monkey kidney cells in a suspension at pH 3.9 became attached to unsensitized starch. In unbuffered saline through which CO_2 had been bubbled, lowering the pH to about 5.0, 20 per cent of Ab-C′ sensitized Pn XIII were attached to washed human platelets, thereby simulating the response shown in Figure 3 (curve 1) for sensitized Pn XIII reacting with chicken E, while in control mixtures only 3 per cent of Ab-sensitized and 1 per cent of unsensitized organisms were adherent. In buffer at pH 3.5, 100 per cent attachment of unsensitized organisms to washed human platelets was observed.

Reactivity of sensitized bacteria or starch granules with these indicator particles has not been seen above pH 5.5 using either washed sensitized antigens or reaction mixtures containing serum and cells from the same species.

B. FURTHER DIFFERENTIATION OF IA AND ACID-ADHESION: QUANTITATIVE STUDIES

1. *Effect of time and temperature of incubation on acid-adhesion and on IA.* Exper. 042057. A suspension of Pn XXXI was sensitized with rabbit Ab and guinea pig C′, washed, and resuspended in saline as described above.

The components of the reaction mixtures for IA and for acid-adhesion were brought to the desired temperature. For acid-adhesion (series A), 0.1 ml. of 2 per cent chicken E in saline were pre-incubated at 37° C. for 20 min. in buffer at pH 3.6 and then brought to the desired temperature. Two-tenths ml. of washed sensitized organisms were then added to the mixtures. Small samples were taken at intervals by capillary pipette and immediately examined by darkfield microscopy. A similar procedure was followed for IA (series B) except that the mixture of human E and SAVB++ was not pre-incubated.

The results are presented in Figure 4. The acid-induced adhesion of sensitized Pn XXXI to chicken red cells was independent of temperature, proceeding very rapidly at 0°, 24°, and 37° C. Identical results were obtained when unsensitized Pn XXXI and chicken cells were incubated together at pH's of 3.0, 3.6, and 4.1.

It was noticed that, if chicken cells and buffer of low pH were mixed at 0° C. without previous incubation at 37° C., the changes usually seen in the cells did not occur and the organisms would not adhere to the unchanged cells over a period of 75 min. After overnight incubation of the whole mixture both the usual changes and the expected degree of adherence were seen. Similar pre-incubation of human E at 37° C. before cooling to 0° and adding antigen did not alter the rate of IA at 0° C. It appears that these changes are necessary for adhesion to occur at low pH, although, as pointed out below, their persistence on raising the pH is not accompanied by adherence. The reason for this is not clear. The essential point is that once they have occurred, the acid-adhesion which follows differs greatly in its kinetics from IA.

IA, on the other hand, is a temperature-dependent process. Adherence occurs rapidly at 37° C. and more slowly at 24° C. At 0° C. the reaction is not only slow but also, over the time range studied, incomplete. These results agree with those previously published[28] and with those of Taverne,[38] who used bacteriophage T2 sensitized with Ab and C′. Taverne suggested that the slower reaction at 0° might be due to a decreased rate of collision between sensitized antigen and red cells. If this were so, it would be expected that the reaction would proceed faster when more concentrated E were used in the reaction mixture, since there would be a greater chance of collision between the bacteria and E. However, in another assay (Exper. 043057), almost identical rates of IA occurred at 0° in reaction mixtures containing 0.1 ml. of 2, 5, 10, and 25 per cent suspensions of erythrocytes. This indicates that the slower reaction at 0° C. is not due merely to mechanical factors.

2. *The effect of salt concentration on IA and on acid-adhesion.* It was thought that the temperature independent acid-adhesion might be due to the formation of an electrostatic bond between oppositely charged particles or sites on the surfaces of the particles, while IA was due to the formation of some other type of bond not primarily electrostatic in nature. Therefore the effect of increasing concentrations of sodium, calcium, and aluminum salts on the formation of the complex and on dissociation of the formed complex was examined in each case. In experiments on IA, however, aluminum could not be used as it caused too much agglutination of human E for microscopic counts to be possible.

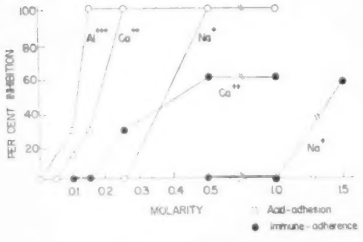

FIG. 5. The inhibitory action of salt concentration on immune-adherence and on acid-adhesion.

Exper. 050657 and 051157. For acid-adhesion two sets of reaction mixtures were made up. In both, 0.5 ml. of buffer, pH 3.2, and 0.2 ml. of 2 per cent chicken cells in saline were incubated together at 37° C. for 20 min. To one was added 0.5 ml. of the salt and 0.2 ml. of a suspension of Pn XXXI; to the other, the bacterial suspension alone. Both were incubated at 37° C. for 30 min.; the second set then received 0.5 ml. of the salt. A similar procedure was followed for IA, except that SAVB++ was used instead of glycine-acetate buffer and saline, and the organisms were sensitized with amounts of Ab and C′ estimated to give 70 per cent adherence to human E.

The results obtained are presented in Figure 5. Moderate concentrations of salts completely inhibited the formation of the bond in acid-adhesion. The degree of inhibition produced by a given salt was in proportion to the charge on the cation. Because of gross damage to chicken cells, sodium and calcium were not tested above 1.0 *M*, but up to this point neither salt dissociated the preformed bacterium-erythrocyte complexes. 0.15 *M* or more concentrated aluminum chloride produced about 25 per cent dissociation. The effect of sodium and calcium chlorides on acid-adhesion of sensitized Pn XXXI to chicken cells at pH 4.1 was similar although a little more marked. The adhesion of unsensitized Pn XXXI was also more sensitive to salt concentration at pH 3.9 than at pH 3.6 or 3.2.

In contrast with the inhibition of acid-adhesion by salts at moderate concentrations, only at relatively high concentrations did sodium and calcium have any effect on the formation of the bond in IA. 1.5 *M* sodium chloride

was required to produce detectable inhibition, while acid-adhesion was completely inhibited by 0.5 *M* salt. Again, 1.0 *M* calcium chloride was only half as effective in inhibiting IA as was 0.25 *M* salt in inhibiting acid-adhesion. Complete inhibition of IA was not produced by any salt concentration, although considerable distortion of cells was caused by the higher concentrations. It is possible that at these concentrations of added salt (e.g. 1.5 *M* or 8.5 per cent sodium chloride) there is some dissociation of Ab and/or C′

TABLE 1. REVERSIBILITY OF THE CHANGE INDUCED IN CHICKEN ERYTHROCYTES (E) BY LOW PH

E treated at pH:	*E incubated with Pn XIII at pH*	*Percentage of organisms adherent*	
		Sensitized	*Unsensitized*
3.5	3.5	100	98
	7.5	16	0
4.0	4.0	94	34
	7.5	8	0
4.55	4.55	100	0
	7.5	0	0
4.9	4.9	28	0
	7.5	2	0
7.5	7.5	0	0

from the antigen. As with acid-adhesion, the bond once formed was not broken at high salt concentrations.

The results of these experiments are considered to support the idea that acid-adhesion is primarily due to the formation of an electrostatic bond, while IA is primarily due to the formation of a bond of some other type.

3. *Reversibility of acid-induced changes in red cells and antigen, and of acid-adhesion.* Exper. 022657. Suspensions of chicken cells were treated with buffers of various pH's for 20 min. at 37° C., washed, and resuspended in buffer of either the original pH or pH 7.5. The cells were then incubated for 30 min. at 37° C. with a suspension in the appropriate buffer of Pn XIII, either sensitized with Ab and C′, or unsensitized. The percentages of bacteria adherent under the various conditions are shown in Table 1.

The change in the red cells induced by low pH and which resulted in adhesion at that pH was reversed by raising the pH. Similarly, exposure of unsensitized Pn XIII to pH 3.5 for 30 min. at 37° C., followed by washing

and resuspension in buffer of neutral pH and incubation with untreated red cells, did not result in adherence.

In another experiment a complex was formed between unsensitized Pn XXXI and chicken erythrocytes at pH 3.2 in which 80 per cent of the bacteria became adherent to cells. The mixture was centrifuged at 2700 rpm for 15 min. (sufficient to spin down all the bacteria as well as the E), washed, and resuspended in VB++ (pH 7.4), and again examined microscopically. Eighty per cent of the bacteria remained adherent during incubation for several hours.

TABLE 2. EFFECT OF TRYPSIN AND OF TANNIC ACID TREATMENT OF ERYTHROCYTES ON IMMUNE-ADHERENCE AND ACID-ADHESION

	Percentage of antigen particles adherent						
Treatment of erythrocytes	*Immune-adherence: Rice starch and human E*	*Acid-adhesion: Pn XXXI and chicken E*					
		pH 3.0	*3.35*	*3.9*	*4.1*	*4.3*	*4.6*
None	88	76	84	72	52	8	0
Trypsin, 0.5%	0	82	84	68	56	16	0
Tannic acid 1:10,000	0	82	80	72	40	12	0

Although the bond once formed persists when the pH is raised, the changes in the erythrocytes and antigen are reversed when treated similarly but separately. The visible structural changes in the erythrocytes do persist, so that these alone are not invariably accompanied by adherence. A reversible change at the sub-microscopic, probably molecular, level, seems indicated.

The complex in IA is not dissociated by quite marked changes in pH since the adherence of washed sensitized *A. cruentus* starch granules is constant from pH 4 to pH 9.

4. *Effect of trypsin and of tannic acid treatment of E on IA and acid-adhesion.* Exper. 042957 and 050157. (a) Trypsin treatment: 1 ml. of a 10 per cent suspension of chicken or human red cells in phosphate-buffered saline, pH 7, was mixed with 4 ml. of 0.5 per cent trypsin (Difco) in the same medium and incubated at 37° C. for 60 min. The cells were then washed three times and made up to a 2 per cent suspension in normal saline. Chicken E were agglutinated moderately by trypsin, but the clumps broke up after the first washing.

(b) Tannic acid treatment: A 2 per cent suspension of chicken or human cells in saline was incubated with an equal volume of 1 : 10,000 tannic acid in saline for 30 min. at 37° C., washed three times, and resuspended in saline.

Assays for IA and for acid-adhesion were carried out in the usual manner with normal and with treated cells. Rice starch granules and *Rickettsia burneti* both sensitized with human "natural" Ab and with human C′ were used in IA assays. Unsensitized Pn XXXI was used in assays of acid-adhesion.

As shown in Table 2, neither treatment had any effect on the range or degree of acid-adhesion. In agreement with our previous results in 1954, the reactivity of human E in IA was abolished both by tanning and by trypsinization. The inhibition of IA by trypsin treatment of the E might thus be due either to the destruction of a receptor or to its masking by the protein enzyme. Whichever occurred, it had no effect on the reactivity of chicken cells in acid-adhesion. Tannic acid treatment is commonly used to render cells capable of absorbing antigens. The reverse effect occurred with IA: the cells became incapable of adhering to sensitized particulate antigens. Since the mechanism of its action is unknown, very little can be deduced from the fact that it inhibits IA. The point to be noted is that these two inhibitors of IA have no effect on acid-adhesion and thus serve to differentiate between the two phenomena.

These results also may be useful in identification of the reactive sites on the surface of primate erythrocytes. Recent assays by Mr. Shaun Ruddy in this laboratory have shown that low concentrations of crystalline chymotrypsin (3 μg.) and of formaldehyde (0.12 per cent) will inactivate or block the reactivity of human E in IA. These collected data are suggestive that the "receptor site" for IA is protein in nature.

DISCUSSION

The experimental results provide several criteria for the differentiation of acid-adhesion of bacteria to other particles from immune-adherence of particulate antigens to primate erythrocytes. These are summarized in Table 3. The effects of changes in salt concentration and temperature on acid-adhesion are indicative that the bond between the particles is electrostatic. As would be expected, the electrostatic attraction varies with the pH and with the species and strain of microorganisms used. Acid-adhesion is also influenced by the presence of Ab and C′ on the surface of the antigen, a point of more practical importance. With the various bacteria studied sensitization with Ab and C′ caused a "shift to the right," i.e., adhesion to

erythrocytes occurred at a higher pH when sensitized bacteria were used than when the bacteria were unsensitized. With Ab and/or C′ from other species of animal it is conceivable that at certain pH's attachment of sensitized bacteria to red cells could occur as a result of either acid-adhesion or IA. Differentiation, as outlined in Table 3, is simple.

TABLE 3. COMPARISON BETWEEN IMMUNE-ADHERENCE AND ACID-ADHESION

Factor	*Acid-adhesion*	*Immune-adherence*
pH	Occurs only below pH 5	Constant over wide pH range (4-9)
Ab and C′	Not essential, but range of adherence is different for sensitized organisms	Essential
Species of erythrocytes	Occurs with all species tested (human, chicken, sheep, rabbit, guina pig)	Occurs with human and monkey erythrocytes only
Other substrates	Platelets (human and rabbit); monkey kidney cells; *E. coli*	Occurs with certain nonprimate platelets only
Time and temperature	Rate of adherence is rapid and constant from 0° to 37° C.	Rate of adherence varies with temperature: rapid at 37°; less rapid at 24°; and slow at 0° C.
Ionic strength	Inhibited by high salt concentration	Not inhibited by high salt concentration
Trypsinizing erythrocytes	No effect	Complete inhibition
Tanning erythrocytes	No effect	Complete inhibition
Previous lysis of erythrocytes	Slight increase in reactivity at high pH's	Human ghosts less reactive than intact human cells. No reaction with lysed nonprimate red cells.

An analogous situation exists with respect to the agglutination of microorganisms by Ab and by auto-agglutination at low pH. The latter phenomenon, acid-agglutination, has been studied by several investigators since 1922.[5, 9, 25, 31, 32] de Kruif[9] found that the optimal pH for auto-agglutination was characteristic not only of the species of bacterium used but often of an individual strain. The addition of immune serum broadened the zone of acid-agglutination.[32] Organisms heavily sensitized with Ab showed a cataphoretic isoelectric point similar to that of γ-globulin. This work suggested

an explanation of our results on acid-adhesion in terms of differences in charge between indicator particle and antigen.

Ponder and Ponder[84] have shown that the isoelectric point of human red cell ghosts is below pH 2.0. The similar ranges of adhesion of sensitized and unsensitized Pn XIII to erythrocytes of all the other species tested indicates that the isoelectric points of these cells are close to that of human erythrocytes. Above this point (pH 2) the surfaces are negatively charged. Below their isolectric points the antigens are positively charged and an electrostatic bond may result. The upper pH limits for adhesion of unsensitized organisms agree quite well with the cataphoretic isoelectric points measured by other workers.[11, 13, 87, 89] Some bacteria do not show reversal of charge below the iso-electric point, e.g., some strains of staphylococci[89] and *S. pullorum*.[13] The failure of *S. typhosa* to adhere when unsensitized may be due to the lack of an amphoteric component on its surface.

That the situation is a little more complex than this is indicated by the decline in adhesion with a further decrease in pH as observed herein with *E. coli, M. albus,* and Pn III (Fig. 1). There are three possible explanations: (a) The positive charge on the bacterium is not very great, and the attraction between bacterium and red cell, initially not strong, declines as the negative charge on the red cell diminishes, until bonds can no longer persist; (b) The bacterium becomes less positive at pH's far below its isoelectric point, as has been described for *B. cereus,*[11] and the attraction becomes too weak for adhesion to occur; (c) It is possible that in all cases the initial attraction is not between oppositely charged particles but between oppositely charged parts of two surfaces with a low net charge of the same sign. Acid-adhesion could thus occur both above and below the isoelectric point of either particle and would depend on the presence of highly but oppositely charged sites on the two particles, each of which had a sufficiently low net charge to allow initial contact.

The shift of the curves in the direction of higher pH when the antigens were sensitized with Ab or with Ab and C′ is probably due to at least partial covering of the surface with protein of higher isoelectric point. The curves did not move as far in the direction of higher pH as might have been anticipated from the isoelectric point of rabbit γ-globulin, pH 5.8 and β-globulin, pH 6.3. Two explanations may be offered for this. First, only a limited amount of Ab was used in sensitizing the bacteria, because agglutination during washing had to be avoided. It is known that very large doses of Ab are required before the maximum acid-agglutination occurs at the isoelectric point of γ-globulin. If the surface is only partly covered with Ab, added C′ seems unlikely to cover much more of the surface, and a similar argument

may be applied. Second, the charge on rabbit γ-globulin is low on either side of the isoelectric point[35] and the positive charge on Ab-sensitized organisms may be too low for attachment to red cells to occur until the pH is well below the isoelectric point of the Ab.

The results of the present study provide little insight into the nature of the attachment of erythrocyte to sensitized bacterium via immune-adherence. The available data indicate that the bond is not electrostatic, although attachment of sensitized bacteria to erythrocytes due to an electrostatic bond can, in the circumstances defined herein, occur. The lack of influence of salt concentration or pH, the influence of temperature on kinetics, and the specific nature of the primate erythrocyte all indicate an immunological reaction based on a complex mechanism the nature of which still remains obscure.

CORRECTION

In the paper entitled "The possible relationship of etiocholanolone to periodic fever," published in *The Yale Journal of Biology and Medicine* (1958, *30,* 395), an error has been found in the chromatograms on page 400. The standard compound D, called "Dehydroisoandrosterone" in Systems 1 and 3 is too nonpolar to be that compound. Subsequent paper chromatography with true dehydroisoandrosterone whose structure has been verified by infra-red spectrum revealed its mobility to be much more polar than the standard etiocholanolone and the original standard "dehydroisoandrosterone."

This error in paper chromatography does not alter the specificity of the identification of etiocholanolone in plasma.

Unfortunately too little of this original standard nonpolar C-19 compound was available to confirm its real chemical structure.

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ON THE MECHANISM OF IMMUNE-ADHERENCE

II. Analogy to Mixed Aggregation of Sensitized Antigens in the Presence of Complement; Immune-Adherence with Animal Platelets

The results in the preceding paper provide strong presumptive evidence that the union of primate erythrocytes (E) to particulate antigens sensitized with both antibody (Ab) and complement (C′) is not caused by simple electrostatic bonds. Further definition of the nature of the attachment has been complicated by the fact that immune-adherence (IA) is strictly dependent upon C′, which is not well defined from a physico-chemical standpoint, and upon primate erythrocytes, the surface of which represents an exceedingly complex substrate.

The need for definitive experimentation is emphasized by the reports of Lamanna and Hollander[20] and of Lamanna[19] in which a number of apparently unrelated particulate substances were purported to substitute for the primate erythrocyte as a substrate or indicator particle for combination with antigens sensitized with Ab and C′. The term serologic adhesion was employed for these various reactions. If substantiated, these observations would simplify considerably the problem of defining mechanism and would indicate that immunologically nonspecific factors were involved. Our experiences over the past four years are not in accord with this concept. Repeated assays with a wide variety of substances consistently yielded negative results.

As a result of a critical examination of our methods and of those cited in reports on serologic adhesion, we were struck by one major divergence in technique. Except in reaction mixtures containing Ab, C′, and erythrocytes from the same individual donor, i.e., an "autologous system," our custom invariably consisted of either: (a) the preliminary absorption of sera used as Ab or as C′ for 30 to 60 minutes with indicator erythrocytes or other in-

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dicator particles; or (b), a washing of the antigen-Ab-C′ complex before mixture with the indicator erythrocytes. Both procedures were designed primarily to avoid blood group antibodies which might react with the indicator erythrocytes, particularly when serum from a heterologous species was employed either as Ab or as C′. Since these precautions were not cited by earlier workers or by Lamanna we developed the hypothesis that certain of their results might be a manifestation of a mixed aggregation of two sensitized particulate antigens joined together by C′. Such a hypothesis would necessitate two assumptions. First, the source of the second Ab, i.e., Ab capable of sensitizing the indicator particle, was presumed to be natural Ab either in the antiserum to the antigen being tested or in fresh serum used as C′. Second, it was postulated that C′ or some component of C′ was polyvalent, i.e., a single molecule or group of molecules could simultaneously attach to sensitized sites on two separate antigens, perhaps in a manner analagous to that involved in systems wherein C′ has been found to influence precipitin reactions.[21, 22, 24]

The experiments cited herein provide initial evidence to support this hypothesis. They clearly demonstrate mixed aggregation which occurs only in the presence of Ab reactive with the indicator particle and in the presence of C′. Similarities of this phenomenon to immune-adherence and to conglutination are cited below. In addition, data are presented which suggest that platelets from certain animals react in immune-adherence similarly to human erythrocytes. As mentioned above, our original studies[28] demonstrated only that human platelets do not substitute for human erythrocytes.

EXPERIMENTAL RESULTS

A. COMPLEMENT AND MIXED AGGREGATION

1. *The requirement for antibody to sheep erythrocytes for reactivity either with particles of an immune precipitate or with sensitized* T. PALLIDUM. Exper. 050457 and 050857. A precipitate of bovine serum albumin and rabbit Ab formed in the equivalence zone was used. The precipitate was washed and resuspended in veronal buffer containing Ca^{++} and Mg^{++} (VB^{++}) so that 1 ml. of the suspension contained about 8.8μg. Ab nitrogen. A 5 per cent suspension of sheep erythrocytes (E) was prepared in VB^{++} to give an O.D. of .680 when diluted 1/15 with water. A portion was diluted ¼ in VB^{++}. A second portion was sensitized with a quantity of rabbit hemolysin estimated to yield about 1000 Ab molecules per cell, washed, and resuspended in VB^{++} to the original concentration, then also diluted ¼ in VB^{++}. Separate samples of both the sensitized (EA) and the unsensitized (E) cells were centrifuged, and the packed cells were lysed by

the addition of distilled water. The stromata were brought back to the original concentration by the addition of a volume of 1.7 per cent saline equal to that of the distilled water used for lysis. These suspensions were also diluted ¼ in VB++. Two aliquots of a pool of guinea pig C′ were used, the first was untreated, and the second was absorbed twice at 0° C. with packed sheep red cells in order to remove the natural sheep hemolysin. The

TABLE 1. THE ATTACHMENT OF PARTICLES OF AN IMMUNE PRECIPITATE (SERUM ALBUMIN-ANTI-ALBUMIN) TO SENSITIZED SHEEP ERYTHROCYTES (EA) IN THE PRESENCE OF DILUTIONS OF FRESH GUINEA PIG SERUM USED AS C′

Assay 1	PERCENTAGE OF PARTICLES ADHERENT *C′ dilutions—unabsorbed serum*						
Indicator particle	*1/1*	*1/2*	*1/4*	*1/8*	*None*	*1/1 heated*	*1/1 with EDTA*
E	36**	34**	4**	0**	0	0	0
EA	84*	84*	20*	6*	2	0	0
E, lysed	36	22	4	0	0	0	0
EA, lysed	88	80	36	0	0	0	0
Human RBC	..	..	..	82	0	0	0

Assay 2	PERCENTAGE OF PARTICLES ADHERENT *C′ dilutions—absorbed serum*					
Indicator particle	*1/1*	*1/2*	*1/4*	*1/8*	*1/16*	*None*
E	0	0	0	0	0	0
EA	74*	98*	36*	10*	4*	0
E, lysed	4	0	0	0	0	0
EA, lysed	96	92	42	14	0	0
Human RBC	..	..	..	..	82	0

* Complete lysis of Ab-sensitized cells by C′.
** Complete or partial lysis by "natural" Ab in unabsorbed serum used as C′.

C′ titers were respectively 208 and 186 $C'H_{50}$ units per ml. The reaction mixtures shown in Table 1 consisted of 0.5 ml. of immune precipitate; 0.5 ml. of C′ dilutions in VB++; and, 0.5 ml. of sheep erythrocytes, i.e., intact or lysed E or EA. Controls were prepared containing heated guinea pig serum and unheated serum plus .01 *M* EDTA. Another set of controls contained 0.1 ml. of washed human erythrocytes in place of the sheep cells. The mixtures were shaken periodically by hand during incubation at 37° C. for 45 minutes. They were then examined by darkfield microscopy and the percentage of precipitate-particles adherent to erythrocytes was counted.

From the results in Table 1 it can be seen: (a) that the adherence of precipitate-particles to sheep cells occurred only in the presence of Ab to the erythrocytes. From the results with unabsorbed versus absorbed C′ it was obvious that the natural blood group Ab in normal guinea pig serum served as a sensitizing substance for the sheep E. The C′ in the mixtures containing EA caused lysis of the sheep cells so that stromata really acted as the indicator particles. In contrast with reactivity in IA with the human erythrocytes, but, for as yet undetermined reasons, only low dilutions of serum as C′ were effective. In contrast to the report of Lamanna and Hol-

TABLE 2. PERCENTAGE OF *T. pallida* ATTACHED TO SHEEP ERYTHROCYTES IN THE PRESENCE OF UNTREATED GUINEA PIG SERUM AND OF GUINEA PIG SERUM ABSORBED WITH SHEEP ERYTHROCYTES

Guinea pig C′	*Human syphilis serum* as Ab (Absorbed with sheep E)*	
	None	*0.1 ml.*
Unabsorbed	2%	66%
Absorbed	0%	0%
Absorbed, plus hemolysin	1%	70%

* The serum was from a patient with latent syphilis diagnosed on the basis of clinical history and a positive treponemal immobilization test.

lander[20] it should be noted that no difference in reactivity occurred between intact and lysed erythrocytes nor between intact and lysed sensitized erythrocytes.

Exper. 060457. As outlined in Table 2, reaction mixtures were prepared containing 0.2 ml. of a suspension of *T. pallidum* in an albumin-phosphate medium; 0.1 ml. of heated serum from a patient with latent syphilis; 0.1 ml. of standardized sheep E; 0.1 ml. of rabbit hemolysin diluted 1/800; and, 0.25 ml. of guinea pig C′. The human syphilis serum was absorbed twice at 0° C. with sheep erythrocytes in order to remove blood group antibody.

After incubation at 37° C. for 60 minutes samples of the various mixtures were examined by darkfield microscopy. Significant adherence of treponemes to the sheep erythrocytes occurred only in the presence of syphilis serum and C′. However, and more significant, no adherence occurred in the mixtures containing about 46 $C'H_{50}$ units of guinea pig C′ which had been absorbed with sheep erythrocytes. Definitive evidence that Ab to sheep erythrocytes is of prime importance in this type of adherence was supplied by the restoration of reactivity upon the addition of rabbit hemolysin to mixtures containing absorbed C′.

2. *The requirement for C′ in mixed aggregation of sensitized* S. TYPHOSA *and sensitized sheep erythrocytes.* Exper. 060557. A suspension of *S. typhosa* 0901 was killed by heating at 65° for 30 minutes, washed 3 times, and resuspended in SAVB++. The suspension was adjusted to an O.D. of .260 at 400 mμ. on the Beckman DU spectrophotometer and then diluted 1/10 for assay. A rabbit antiserum against *S. typhosa* (agglutination titer: 12,800 units per ml.) was diluted 1/1000 in SAVB++. Sheep erythrocytes were prepared as above and one portion was sensitized with an equal

TABLE 3. THE REQUIREMENT FOR COMPLEMENT IN MIXED AGGREGATION OF SENSITIZED SHEEP ERYTHROCYTES (EA) WITH SENSITIZED *S. typhosa*

Erythrocytes	*Guinea pig serum absorbed twice with erythrocytes (E)*	*Percentage of* S. typhosa *adherent to erythrocytes with dilutions of guinea pig serum*						
		1/1	*1/2*	*1/4*	*1/8*	*1/16*	*1/32*	*None*
Intact EA	Untreated*	80	86	84	46	10	0	0
Intact EA	Untreated, plus EDTA†	0	0	0	0	0	0	0
Intact EA	Decomplemented	0	0	0	0	0	0	0
Lysed EA	Untreated, plus EDTA†	0	0	0	0	0	0	0
Intact E	Untreated‡	0	0	0	0	0	0	0
Lysed E	Untreated	0	0	0	0	0	0	0

* Complete lysis of the sensitized erythrocytes occurred in all mixtures containing dilutions of C′.
† No lysis of EA occurred in the presence of EDTA.
‡ No lysis of E occurred in any reaction mixture.

portion of rabbit hemolysin diluted 1/800. A sample of the nonsensitized cells was mixed with distilled water so that about 90 per cent of the cells were lysed. Guinea pig C′ which had been absorbed twice with sheep erythrocytes as above was diluted in SAVB++. Dilutions were also prepared of one sample of C′ which had been "decomplemented" by treatment at 0°C. for 24 hours with the washed precipitate from a mixture of bovine serum albumin and rabbit antibody. This treated serum was not reactive in the usual hemolytic assay.

Reaction mixtures containing 0.2 ml. bacteria; 0.2 ml. of rabbit anti-typhoid serum; 0.1 ml. sheep E; 0.1 ml. rabbit hemolysin; and 0.2 ml. of C′ dilutions were prepared as in Table 3. By darkfield microscopy the percentage of bacteria adherent to erythrocytes was measured after incubation for 60 minutes at 37° C.

The identification of C′ as an essential substance for mixed aggregation in these reaction mixtures was strongly implicated. No mixed aggregation

occurred with the guinea pig serum which was "decomplemented" by treatment with an immune precipitate. Similarly no aggregation occurred in the mixtures containing .001 *M* EDTA which binds Ca^{++} and Mg^{++} and hence inhibits $C'_{1,4}$ and C'_2.

Furthermore, no attachment of sensitized bacteria to either intact or lysed sheep erythrocytes occurred in the presence of guinea pig C′ which had been absorbed to remove naturally occurring agglutinins for sheep erythrocytes. This result agrees with the preceding observations with washed immune precipitates and with *T. pallidum* (Tables 1 and 2), and with our previously published results.

3. *A role of auto-antibody in IA.* In view of the fact that mixed aggregation of two sensitized antigens requires C′, we have considered the possibility that immune-adherence could be explained on the assumption that human erythrocytes have small amounts of auto-antibody on their surface. Attempts to identify Ab on the surface of human red cells as the "receptor site" have been unsuccessful to date.

First, attempts were made to dissociate the hypothetical Ab. Six-tenths ml. of packed normal human erythrocytes was washed three times with saline and resuspended in 30 ml. of distilled water. Two portions of 15 ml. were washed twice with distilled water. After high-speed centrifugation, the deposit from one batch was resuspended with 10 ml. of 15 per cent NaCl. The deposit from the second batch was resuspended with 10 ml. of acetate buffer, pH 3.0. Both samples were incubated for 60 minutes at 37°, washed, and brought to 10 ml. with $SAVB^{++}$. These preparations were divided and mixed with a sample of washed presensitized yeast cells and with a sample of yeast cells mixed with serum as Ab and C′. While readings were difficult because the stromata were badly distorted, about 40 per cent of red cells pretreated either with hypertonic NaCl or at low pH, were observed to be attached to the sensitized yeast. No difference was noted between the washed and unwashed antigen.

Second, in view of the possibility that auto-antibody might develop late in humans, erythrocytes from young children were examined. Blood was collected in modified Alsever's solution from 34 children between the ages of 8 days and 10 years. Greater than 78 per cent of the washed erythrocytes from each individual reacted in immune-adherence with washed typhoid bacilli presensitized with Ab and C′.

B. THE REACTIVITY OF NONPRIMATE PLATELETS IN IMMUNE-ADHERENCE

Exper. 071157. The reactivity of platelets as indicator particles in IA was examined using sensitized sheep erythrocytes as the particulate antigen. Platelets were isolated from fresh blood of human beings and guinea

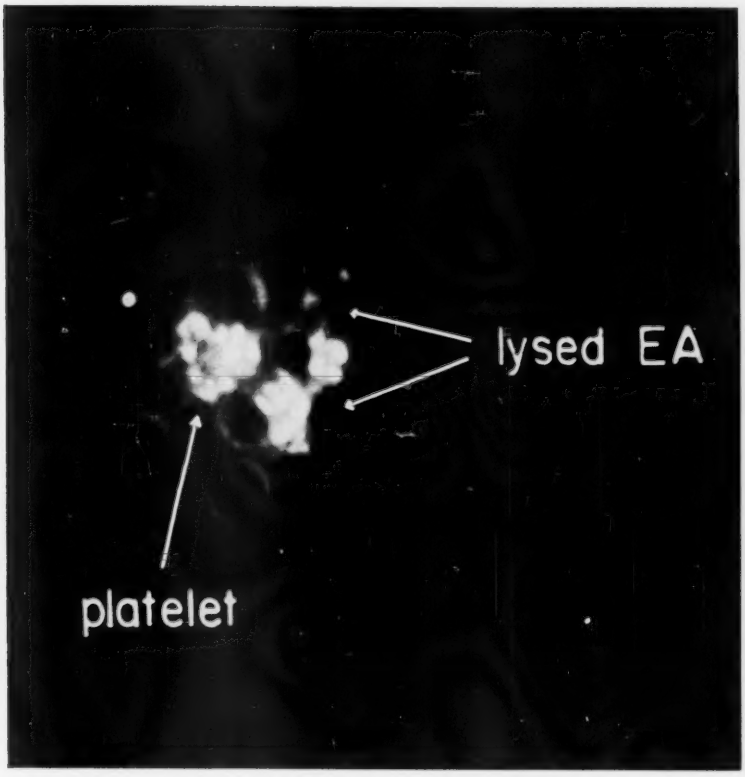

FIG. 1. Photomicrograph of immune-adherence between sensitized sheep erythrocytes (EA) and washed guinea pig platelets. The sheep erythrocytes are lysed due to reactivity with C'. Three platelets are visible in the photograph.

pigs. Five ml. of whole blood were mixed respectively with 5 ml. of cold Alsever's solution, 5 ml. of cold .006 *M* EDTA in veronal buffer, and with approximately 2 ml. of washed resin (IRC-50 in the Na^+cycle). All mixtures were centrifuged at 0° C. for 30 minutes at 600 rpm. The supernatant fluids contained platelets and plasma were diluted with 5 ml. of Alsever, EDTA, and veronal respectively and were washed three times by centrifugation at 2000 rpm for 20 minutes. The final deposit was re-

TABLE 4. ADHERENCE OF GUINEA PIG PLATELETS TO SENSITIZED SHEEP ERYTHROCYTES IN THE PRESENCE OF DILUTIONS OF FRESH HUMAN AND GUINEA PIG SERUM AS C'

Platelets	*Method of isolation*	*Serum used as C'**	*Percentage of platelets adherent with dilutions of C'*				
			1/1	*1/4*	*1/16*	*1/64*	*None*
Human	Alsevers	Human	0	0	0	0	0
	.003 *M* EDTA	Human	0	0	0	0	0
	IRC-50 resin	Human	0	0	0	0	0
Human	Alsevers	Guinea pig	0	0	0	0	0
	.003 *M* EDTA	Guinea pig	0	0	0	0	0
Guinea pig	Alsevers	Human	20†	58	92	86‡	0
	Alsevers	Guinea pig	95	95	95	95	0

* Complete lysis of EA with human C' 1/1 and 1/4; partial lysis with 1/16. Complete lysis of EA with guinea pig C' 1/1, 1/4, 1/16; partial lysis with 1/64.

† Distortion of platelets was noted microscopically.

‡ Only about 5 per cent of EA was lysed with 1/64 human C'. The majority of platelets were attached to intact erythrocytes indicating that lysis is not a prerequisite for adherence.

suspended in $SAVB^{++}$ and centrifuged at 400 rpm for 5 minutes to remove the few erythrocytes and the rare clumps of platelets. The supernatant fluids containing only platelets were standardized to 50 per cent transmittance on the Beckman DU spectrophotometer at a wave length of 400 mμ. Reaction mixtures were prepared as follows: 0.2 ml. of $SAVB^{++}$; 0.1 ml. of EA washed and standardized as usual to an O.D. of .340 at 541 mμ; 0.2 ml. of standardized platelets in $SAVB^{++}$; and, 0.1 ml. of dilutions of fresh autologous serum as C'. The latter serums were obtained from clotted blood drawn at the same time as the blood used to isolate platelets.

All mixtures were shaken intermittently during incubation at 37° C. for 45 to 60 minutes. Samples were withdrawn and examined by darkfield

microscopy for the percentage of platelets attached to the sensitized particulate antigen EA. The lysis of EA by C′ was recorded.

Certain typical results are shown in Table 4, and an example of adherence of platelets and EA is shown in Figure 1. In the presence of fresh serum, the sensitized particulate antigen, EA, became attached to about 95 per cent of the guinea pig platelets. No adherence of human platelets occurred despite the proved combination of the human C′ with the particulate antigen as measured by lysis of the EA. Nor did adherence occur with human platelets in the presence of guinea pig C′. An inhibition of reactivity of guinea pig platelets occurred with low dilutions of human serum. The mechanism of this inhibition is not clear.

Because of the striking difference of reactivity of guinea pig and human platelets this type of assay has been repeated. To date, platelets have been isolated as outlined above from 25 human beings. In reaction mixtures containing either human C′ or guinea pig C′ all samples were nonreactive with sensitized sheep erythrocytes, sensitized starch granules, and sensitized typhoid bacilli. This lack of reactivity agrees with our original observation that human platelets do not react in IA, and stands in contrast with a recent report that human platelets react equally well with sensitized *T. pallidum* in what was termed serologic adhesion.[19, 20] Of greater interest, however, are the results with guinea pig platelets. The reaction between EA and guinea pig platelets is particularly striking even with 0.1 ml. of C′ diluted 1/64, which is about the same level of reactivity of C′ in immune-adherence involving human erythrocytes as indicator particles. In order to classify this reaction as IA or C′-dependent mixed aggregation and to exclude a possible role of blood-coagulation factors in this reaction, additional experiments were performed.

1. Platelets from 12 monkeys *(M. rhesus)* failed to react with a variety of sensitized antigens in the presence of either autologous serum or pooled homologous serum as C′. Erythrocytes from all 12 monkeys reacted in IA with sensitized typhoid bacilli with autologous serum as C′.

2. Rabbit platelets reacted with EA as the particulate antigen in the presence of autologous C′.

3. Guinea pig platelets were incubated with autologous serum for 20 minutes at 37°, washed, and then mixed with EA. No mixed aggregations resulted.

4. Suspensions of rice starch and of *S. typhosa* were sensitized with Ab only and with Ab and C′. The suspensions were washed three times in SAVB++ and samples were mixed with washed human erythrocytes and washed guinea pig platelets. Approximately 70 per cent of both the starch granules and the typhoid bacilli which had been sensitized with Ab and

C′ became attached to the human erythrocytes and to the guinea pig platelets. No attachment to the indicator particles occurred with untreated starch or typhoid bacilli, or with these antigens when sensitized only with Ab. Since no free serum was present in the reaction mixtures containing the sensitized and washed antigens and the platelets, these results are interpreted as being due to immune-adherence, and not as a reflection of some factor involved in the blood coagulation system, or of some factor liberated in serum as a result of reaction of the antigen-antibody complex with C′.

5. The products of lysis of sheep E and EA by water did not induce adherence of EA with guinea pig platelets in mixtures lacking C′.

6. The adherence of platelets to some unlysed EA at high C′ dilutions (Table 4) was confirmed in two additional trials.

7. Recent assays performed in collaboration with Dr. Maria Siqueira have demonstrated that the soluble O antigen of *S. typhosa,* anti-typhoid serum, and guinea pig C′ induce striking agglutination of guinea pig platelets. This agglutination response appears to be analogous to hemagglutination of human erythrocytes caused by IA with either particulate or soluble antigens.[20] No agglutination has occurred in reaction mixtures containing human platelets.

DISCUSSION

The results obtained herein are interpreted to mean that mixed aggregation of two sensitized particulate antigens may occur in mixtures containing complement. At least three factors of major consequence have evolved.

First, insight has developed into the mechanism of serologic adhesion as reviewed by Lamanna.[19] It seems likely that certain of the experiments described by this investigator were misinterpreted as immune-adherence due to an oversight of natural antibody to the "indicator particle" in the serum used as a source of C′ or in the serum used as Ab to *T. pallidum.* The presence of natural Ab capable of inducing immune-hemolysis in the presence of C′ would of course explain the peculiar circumstance wherein only lysed animal erythrocytes were stated to be reactive in adhesion with sensitized *T. pallidum.* Therefore, we are left with the conclusion that C′-dependent mixed aggregation and immune-adherence are probably separate phenomena and that both are different from acid-adhesion as outlined in the preceding paper. The differentiation of IA from mixed aggregation is not definitive, however, in that the bond in IA remains obscure. It is theoretically possible that primate erythrocytes are sensitized *in vivo* with blood group Ab, which is not removed by repeated washing *in vitro.*

Second, platelets from certain animal species react with sensitized particulate antigens in the presence of C′. Because of the similarities of this reactivity of nonprimate platelets to immune-adherence with primate erythrocytes, it is postulated that the two reactions represent similar mechanisms, and, for the present, both are termed immune-adherence. It is of considerable interest that a sharp differentiation in behavior of erythrocytes versus platelets occurs in different animal species. Primate platelets have been consistently nonreactive while among erythrocytes, those from primates have been the only reactors of any significance. It seems clear that immune-adherence with human erythrocytes as here defined is identical with the

TABLE 5. SCHEMATIC REPRESENTATION OF THE BOND INVOLVED IN C′-DEPENDENT MIXED AGGREGATION

$$G_1 \cdot A_1 - C' - A_2 : G_2$$

or

$$G_1 \cdot A_1 \cdot C' - Cg - C' \cdot A_2 \cdot G_2$$

$G_1 \cdot A_1$ represents a particulate antigen (G) sensitized with its specific antibody (A).
$G_2 : A_2$ represents a second particulate antigen, e.g. the indicator particle, sensitized with its specific antibody or with natural antibody.
Cg conglutinin

"red cell adhesion" described by Duke and Wallace[10] in 1930. Similarly, certain examples of the Rieckenberg adhesion phenomenon between sensitized leptospira or trypanosomes and rat platelets may well represent IA, but there is serious doubt that adhesion to other substrates such as lysed non-primate erythrocytes, primate platelets, bacteria, etc., represents the same phenomenon. Instead, our results would suggest that reactions with the latter substances are due either to C′-dependent mixed aggregation, or under certain circumstances, to acid-adhesion.

Third, the necessity for a clear interpretation of the mechanism of C′-dependent mixed aggregation is emphasized by the possible role of conglutinin in the experiments herein. Conflicting concepts of conglutinin have been offered by different investigators,[12, 14, 40] but perhaps the most comprehensive description of this substance and its role in immunological phenomena may be found in the reports of Coombs and associates.[3, 8, 9, 14] Using the terminology of the latter, it is conceivable that conglutinin is the agent in guinea pig serum which causes mixed aggregation in the presence of C′ by a mechanism schematically outlined in Table 5.

Regardless of the interpretation as to mechanism it seems obvious that mixed aggregation might well serve as a method of detection of Ab to a

particular microbe or tissue particle. However, it seems equally obvious that precautions should be taken to use optimally sensitized "indicator particles" rather than to depend upon often variable (and occasionally unrecognized!) natural Ab in the serum used as C′. There are, however, certain theoretical disadvantages, particularly in the fact that only low dilutions of guinea pig or human serum suffice as a source of C′ or of conglutinin.

Considerable emphasis is given to the necessity for additional definitive experimentation upon the substances responsible for the reactivity of normal sera with a variety of bacteria and other particulate antigens. As outlined herein the responsible factors are serum proteins which require complement for their reactivity. Hence they are termed "natural" antibody. In addition to their role in the above experiments their potential importance in other biological systems has been indicated in parallel experiments[37] which have resulted in the concept that the phenomena ascribed to properdin in normal serum are not due to a single entity but instead are caused by natural antibody acting in conjunction with three of the components of complement, i.e., C'_1, C'_4, and C'_2. While there is ample evidence that some natural antibodies show rather broad cross-reactivity, associated experiments in this laboratory have demonstrated an immunological specificity of the natural antibodies involved in neutralization of different bacteriophages (T series) of *E. coli*,[4] and of the natural antibodies which react with starch granules and with zymosan (yeast cell wall) particles.[41]

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STUDIES ON THE PATHOGENESIS OF VASCULAR DISEASE

Corneal Connective Tissue—Plasma Lipid Interactions**†

That diet and the state of the blood lipids are in some way related to the establishment of arteriosclerosis is supported by voluminous epidemiological, clinical, and experimental evidence, but the exact roles of these factors in the pathogenesis of this important disease have not been clarified.

In this laboratory, experiments have demonstrated that lipids from the ambient plasma, derived either from chylomicrons or from lipoproteins, localize and accumulate selectively at the sites of acute inflammatory lesions experimentally produced in the coronary arteries of animals.[14, 15] It was emphasized that such localization of lipids is dependent, within wide ranges of blood lipid levels, upon the presence of the inflammatory arterial foci. With certain lipids and in certain circumstances, transformations of the basic arterial lesions to fatty, foam-cellular granulomas, similar to those of arteriosclerosis, have been observed. However, acute inflammatory foci in muscular vessels such as the dog's coronary arteries can hardly serve as pathogenetic models for the intimas of the elastic arteries of man, in which arteriosclerosis preferentially develops. The living cornea has seemed to be a more suitable site for the study of connective tissue-lipid interactions of interest in the pathology of chronic vascular disease.

Background exists for such an approach. In the course of studies of mustard gas injuries of the eyes in men and in experimental animals, Mann and Pullinger[8, 9] in 1942 called attention to occasional deposits of cholesterol and of other lipids in the corneal lesions. These deposits were associated with vascularization from the limbus and were often accompanied by hemorrhage or by ulceration. Mann and Pullinger suggested that certain of the lipid-connective tissue reactions occurring in the cornea resembled those in

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the arterial intima in atherosclerosis. Cogan and Kuwabara[2, 3, 4] have been interested in the cornea as a site for the study of intracellular lipid synthesis and its possible relation to atherogenesis. Recently (1958)[5] these investigators have produced fatty intracorneal lesions by combining corneal injury (thermal) and cholesterol feeding in rabbits.

Investigation of the reactions of the cornea *in vivo* to injected plasma lipids was begun in this laboratory in 1955, and preliminary reports of many of the observations that follow have been published.[11-13, 16] The present paper describes the experiments and the results in greater detail.

MATERIALS AND METHODS—GENERAL STATEMENT

In general the morphological reactions of dogs' or rabbits' corneas to intracorneally injected plasma lipids and to other pertinent materials have been studied at various time intervals after injection.

Healthy adult dogs and albino or Dutch strain rabbits maintained on standard, low-fat laboratory rations were utilized. They were prepared for intracorneal injection by intravenous Pentothal or Nembutal anesthesia, after which the conjunctival sacs were copiously irrigated with physiological saline solution. Injection was made at moderate pressures into the central area of the cornea through a No. 27-gauge hypodermic needle. The amount of sterile serum or other material injected varied from 0.05 ml. to 0.15 ml.

Control materials injected intracorneally included physiological saline, clear serum, or plasma from fasting dogs and rabbits, lipids extracted by ether from human serum, and viosterol in oil.

In the experimental series, corneal reactions to chylomicrons, to chylomicron-free opalescent human serum and plasma, to chylomicron-free opalescent animal serum after cholesterol feeding, and to clear human serum and plasma fractions and clear animal serum fractions after cholesterol feeding were tested. Also tested in a preliminary fashion in the cornea were human lipoprotein concentrates, denatured serum lipoproteins, and plasma without anticoagulant from intact animals and from renal-artery-ligated dogs.

Methods of preparation of control and experimental materials used for injection are given in detail in appropriate sections of the report.

During the course of the experiments, the eyes were carefully observed for changes in the gross appearance of the injected corneas. Depending upon the objective of the experiment, animals were sacrificed at intervals varying from a few minutes to three months after injection and their corneas were removed for histological study. Fixed in 10 per cent neutral formalin, frozen sections were stained with sudan IV for tissue lipids. Paraffin sections stained with hematoxylin and eosin, by Masson's method or by Mallory's phosphotungstic acid technique for fibrin, were examined, often in parallel with the fat stains. Schultz reactions for tissue sterols were also carried out on frozen sections.

Procedures for determination of serum lipid fractions were those in regular use in this laboratory.[15]

CONTROL INJECTIONS

Microscopic examination of many control, normal dog, and rabbit corneas revealed no sudanophilic material. The insertion of a 27-gauge needle into the central area of the anesthetized dog's or rabbit's cornea produced a small lesion confined to the area penetrated. In the first few days there was necrosis with some cellular exudate limited to the vicinity of the needle tract. By one week, only a small, localized, fibrous scar remained, often with epidermidization of the tract itself. No vascularization or lipid deposit occurred. Likewise, injections of sterile physiological saline solution or of sterile, clear, chylomicron-free dog or rabbit serum or plasma from animals on low-fat diets led to a nonvascular, needle-tract scar of approximately the same size. The injection of these control substances rendered the cornea immediately edematous and opaque. The saline-injected corneas cleared rapidly, except for the scar of the needle tract, usually within 24-48 hours, whereas the serum-injected eyes took a day or two longer. No stainable lipid was present in any of these lesions microscopically and little change in collagen fibers was observed.

Occasionally there were breaks in sterile technique. When this occurred with any of the materials injected, there ensued great edema of the cornea, lasting opacity, large zones of necrosis, accumulation of polymorphonuclear leucocytes, and abscess formation leading often to corneal ulceration. Little difficulty was encountered in distinguishing such reactions from those associated with the presence within the cornea of sterile plasma or its components. It was noted, moreover, that sudanophilic granules were regularly present in the cytoplasm of polymorphonuclear leucocytes if these appeared in the cornea. Unless infection was present, needling of the cornea or the injection of physiological saline or of normal dog or rabbit serum or plasma did not result in vascularization. Of interest was the observation that fresh human or dog plasma without anticoagulant *did not* clot when placed intracorneally in dogs. This phenomenon will be mentioned further in the section describing the results of intracorneal injections of plasma from renal-artery-ligated dogs.

In anticipation of studies of the interactions of corneal tissue and the lipids in native plasma, preliminary studies on certain other lipids were carried out. Viosterol (100,000 units vitamin D per ml.) in corn oil was injected into six dog corneas. This oily material remained in the cornea in droplet form. Over a three-week period it elicited no reactive response in the corneal tissue, although loss of corneal stromal nuclei occurred in the infiltrated areas (Figs. 1, 2).

Six dog corneas were also injected with the lipids extracted by ether from human ACD blood-bank plasma. This semisolid fatty substance was observed in the corneas for six weeks. After injection the eyes cleared but subsequently became vascularized. Around the lipid mass in the cornea there was necrosis, fibrosis, and much phagocytosis of fat. Acicular crystals often appeared within the lipid masses.

CORNEAL REACTIONS TO INJECTED CHYLOMICRONS

Chylomicrons were obtained from dog serum after a meal of olive oil (100 ml.) and horse meat (0.5 lb.). A representative sample of such serum contained: total cholesterol 99.6 mg.%, free cholesterol 33.6 mg.%, lipid phosphorus 12.2 mg.%, and fatty acids 29.6 mEq./1. The serum was centrifuged at 5,000 r.p.m. for one hour in the preparative ultracentrifuge. After centrifugation the upper cream layer (chylomicrons) was pipetted off and was injected as such, or was washed two or three times in sterile saline and recentrifuged before injection. These particles, before or after centrifugation, were visible in the low power of the microscope and cleared completely and rapidly upon incubation with heparinized dog plasma.

Chylomicrons from human blood were prepared by similar centrifugation of blood-bank plasma or from freshly drawn lactescent blood after a fatty meal. They were injected intracorneally as saline-washed particles or in their native medium, the effect of their presence in the cornea being evaluated by comparing corneal reactions to whole, hyperlipemic plasma or serum with those following injection of the same plasma or serum after centrifugation.

Seventy-eight corneas injected with 0.05 to 0.15 ml. of one of these preparations have been examined at intervals up to three months following injection (Figs. 3, 4, 5, 6, 7, 8). Results generally did not vary with either hyperlipemic dog or human serum or with washed chylomicrons from either source suspended in physiological saline. With the latter preparations the impression was gained that larger lipid droplets were present at all stages in the cornea and that there was slightly more ingestion of lipoid material by phagocytes.

Directly following injection, chylomicrons from dog or human serum could be observed microscopically as minute sudanophilic droplets among the lamellae of the edematous cornea. These droplets did not differ in appearance from chylomicrons in stained spreads of the original material. After several days, agglomeration of much of the injected lipid had taken place, resulting in large, intensely staining sudanophilic droplets and irregularly shaped fatty masses in the infiltrated zones. No cellular exudate was

present at this time, and the cornea was no longer edematous. After three to six weeks in nonvascularized corneas, at the margins of the lipid pool, small accumulations of lipophagic mononuclear cells appeared. It could not be determined with certainty whether these represented proliferation *in situ* of the corneal stromal cells or whether these cells represented mononuclear phagocytes from the limbus. Changes in the corneal stroma occurred progressively in association with the deposited lipids. The lamellar collagenous fibers appeared to swell and to fuse into broad pale-staining, homogeneous structures. These were stained diffusely with sudan dyes. The nuclei of stromal cells had disappeared in the zones so involved.

The above observations followed injection of large concentrations of chylomicrons into the cornea. If smaller concentrations were injected, for instance, 0.05 ml. of mildly lactescent (2+) dog serum, both dog and rabbit corneas cleared almost completely within two to three weeks, and microscopically demonstrable lipid disappeared. An additional observation of interest was made. If a chylomicron-rich serum was cleared *in vitro* by heparinized plasma and was then injected intracorneally, no sudanophilic residue remained in the cornea.

In summary, serum chylomicrons within the cornea elicited only a mild peripheral lipophage reaction together with fusion and homogenization of corneal collagen within the lipid pool. Upon injection of small quantities there was a marked tendency for chylomicrons to disappear without eliciting corneal reaction.

With the advent of vascularization and the ingrowth of vascular pannus from the limbus of the eye, striking changes in the character of the lesions associated with chylomicrons occurred (Figs. 9, 10). Vascularization took place, it should be pointed out, at variable times after injections of different lipids, but appeared to be related more to the size of the injected lipid pool and to its position rather than to the type of plasma lipid injected. After the injection of chylomicrons from dog or human serum, vascularization usually took place within three to four weeks. The corneal tissue between the limbus and the intracorneal lipid pool became cloudy and edematous and fine capillary loops reaching toward the central area of the cornea could be visualized. As these approached the edge of the lipid pool, gross hemorrhage occurred frequently from the leading capillary buds. Fibroblasts, mononuclear cells, and scattered polymorphonuclear cells accompanied the capillary ingrowths. Often the zone of contact of the ingrowing pannus with the connective-tissue lipid pool became even more edematous, necrosis of tissue occurred, and ulceration ensued. In contrast, without vascularization, ulceration of the

cornea after injection of chylomicrons was rare unless infection had supervened.

As the vascular and fibrous pannus grew into the center of the cornea, active phagocytosis of the lipids and of the altered lipid-impregnated collagen of the lipid-injected area occurred. The lipophagocytosis was accomplished by large mononuclear cells and by spindle cells of fibroblast type. The result of these cellular activities, a week or so after vascularization, was the transformation of the area into a fatty, xanthomatous inflammatory granuloma, associated frequently with hemorrhage or ulceration of the cornea. It should be re-emphasized that such changes did not occur before vascularization, the zone of the lipid pool remaining quiescent except for the mild marginal lipophagocytosis already described.

As vascularization of the intracorneal lipid-pool area progressed, it was noted that lipid disappeared from the zones earliest involved. Microscopic investigation of lesions of various ages indicated that the intracellular lipid of the spindle-cell lipophages of the pannus became progressively less, accounting for the observed depletion of sudanophilic materials. The final result of the ingrowth of pannus from the limbus, if ulceration did not take place, was dense corneal scar with no or little residual lipid. The attainment of this stable state usually took from three to six weeks from the commencement of capillary ingrowth from the limbus.

CORNEAL REACTIONS TO INJECTION OF SERUM OR PLASMA

Preparation of serum and plasma and their derivatives for intracorneal injection must be given in some detail because of differences in the physical state and distribution of lipids in the various materials. All serum was initially separated from clot by low speed centrifugal spins of 3,000 r.p.m. for 15 minutes. Clear serum from dogs or rabbits on low-fat diets was spun at 5,000 r.p.m. for one hour to remove any microscopically visible particles. (16 mm. objective—10 x ocular, reduced transmitted light). After a large olive oil-meat feeding, lactescent dog serum was obtained, which was freed of microscopically visible particles by a spin of 5,000 r.p.m. for one hour. The subnatant layer of such postprandial centrifuged dog serum was limpid —without trace of opalescence. After two to four weeks of cholesterol feeding in dogs or rabbits, lactescent sera were obtained. These contained microscopically visible particles which contributed to, but which were not responsible for, much of the lactescence of the serum. Such particles together with other lactescent materials were removed in the top serum layers after centrifuge runs of 5,000 r.p.m. for one hour. The subnatant layers of these sera from cholesterol-fed animals were often strongly opalescent but

contained no particles resolved at 160 diameters in transmitted light. These opalescent sera did not clear upon incubation with heparinized plasma *in vitro,* and were considered, as Gofman[9] has pointed out, to contain lipoprotein lipids including many low density classes.

If opalescent, chylomicron-free sera from cholesterol-fed animals were now spun at 30,000 r.p.m. in the preparative ultracentrifuge at the specific gravity of serum, a turbid, creamy top layer was separable from the nearly always limpid bottom layer. The top, lactescent layer dispersed in 1 ml. of its own subnatant fluid contained no microscopically visible particles and was not cleared by incubation with heparinized plasma. It was considered to contain large quantities of low-density lipoproteins contributing to the originally observed lactescence. The clear, bottom layers of sera of cholesterol-fed animals after spins of 30,000 r.p.m. for an hour or more contained residual lipoprotein.

In summary, limpid chylomicron-free sera could be obtained from animals on low-fat diets by centrifuge runs of 5,000 r.p.m. for one hour. Clear, chylomicron-free serum from dogs after a single olive oil-meat feeding could be obtained by similar low-speed centrifugation. With cholesterol feeding in animals, chylomicrons, but not opalescence, could be removed by centrifuging serum at 5,000 r.p.m. for one hour. Centrifugation at 30,000 r.p.m. for one hour or more at the specific gravity of serum resulted in a turbid, fatty top layer, in which the materials responsible for the opalescence were concentrated, and a clear subnatant fraction which contained residual lipoprotein lipids.

Human serum for intracorneal injection was obtained from healthy adult donors in the post-absorptive state, or after mixed fat-containing meals. Blood lipid levels in these donors were known to be within the usually accepted normal range. Human plasma was obtained as blood-bank plasma or from recently outdated ACD bank blood. Low speed centrifugation (5,000 r.p.m. for one hour) of relatively clear postabsorptive serum or plasma removed microscopically visible particles. These particles were cleared by heparinized plasma *in vitro.* Faint opalescence occasionally remained in even these samples, and in originally lactescent sera or plasma opalescence was often prominent after microscopically visible particles had been removed. There was great variation in the amount of this residual opacity among postprandial sera or plasmas from different individuals. Subsequent centrifugation at 30,000 r.p.m. for one hour or more concentrated the lactescent materials in the top layers, leaving clear, nonopalescent subnatant fractions.

There were thus available for injection from human serum or plasma, as in the cholesterol-fed animals, a chylomicron-rich fraction, an opalescent fraction rich in low density lipoproteins, and a clear subnatant fraction containing residual lipoproteins.

Native, post-absorptive, grossly clear plasma without anticoagulant was prepared, free of visible microscopic particles, by centrifugation at 10,000 r.p.m. for 5 minutes in siliconized or paraffined tubes with all equipment at 4° C.

Corneal reactions to injected chylomicrons have already been described. Approximately 200 corneal lesions resulting from injection of serum or serum derivatives have been studied. Representative lesions are illustrated in Figures 11-18.

The injection of small amounts of sterile, clear autologous or homologous dog serum (from animals on low-fat, control diets) into the central areas of dogs' corneas resulted only in transient corneal edema, as has been mentioned. Within three to four days after injection, the corneas were clear without microscopic residue except for the scar of the needle track. Similar results followed the injection of like amounts of clear, control rabbit serum into dogs' or rabbits' corneas.

The intracorneal reactions in dogs and rabbits that followed the injection of small quantities of chylomicron-free serum from hypercholesterolemic rabbits set a pattern that was observed also with serum from cholesterol-fed dogs and with many concentrates of human serum and of human serum lipoproteins.

Rabbits were fed raw egg-yolk mixed with chopped lettuce or with rabbit pellets (Purina rabbit checkers—fat 2 per cent). After two to four weeks of such feeding, the animals' sera were lactescent with greatly elevated serum lipid levels. Serum cholesterols ranged from 300-3,000 mg.%. Low speed centrifugation (5,000 r.p.m. for one hour) removed microscopically visible particles, but not opalescence. Indeed, all opalescence was not removed in some samples following spins of 30,000 r.p.m. for several hours at the specific gravity of plasma. The opalescent, chylomicron-free serum has been injected into a series of 30 dog corneas and 26 rabbit corneas, and has been observed over a four-week period. Initial transient opacity and swelling of the cornea resulted just as it had following the injection of control serum. After edema subsided, a faint hazy zone corresponding to the position of the injected serum droplet remained. Microscopically, an intense sudanophilia of the corneal stroma persisted. Over a two-week period gradual increase in opacity of the cornea occurred, limited exactly to the area of the original serum infiltration and corresponding to the microscopically ob-

served diffuse lipid deposits. This opaque area was not due to corneal edema, and it was separated from the limbus by a completely clear corneal zone. Microscopic examination of these corneas at successive intervals revealed that the observed opacities were associated with progressive fusion and homogenization of the corneal stroma and with active phagocytosis of lipid and of swollen lipid-impregnated corneal fibers. Lipophages apparently derived from fixed tissue cells in the area, as no proliferation or infiltrative cellular activity could be observed in the clear zones between the developing lesion and the limbus. Also there was increase of spindle cells of fibroblast type in the area. Thus, in dogs' and rabbits' corneas, foam-cellular xanthomata developed as a result of the intracorneal injection of chylomicron-free, opalescent, cholesterol- and lipoprotein-rich rabbit serum. The reaction was independent of vascularization from the limbus.

The material in this serum responsible for opalescence could be concentrated by centrifuge runs of 30,000 r.p.m. for one hour. Such a concentrated sample of rabbit serum contained: total cholesterol 440.0 mg.%, free cholesterol 223.0 mg.%, lipid phosphorus 25.0 mg.%, and fatty acids, 88.0 mEq./l. Dispersed in its own subnatant fluid no microscopically visible particles were present. Injected into dogs' or rabbits' corneas, intense sudanophilia remained after subsidence of edema, and even more clearly recognizable fatty intracorneal granulomas occurred than those following injection of unconcentrated opalescent serum. With the clear, subnatant fraction of serum from cholesterol-fed rabbits after 30,000 r.p.m. spins, sudanophilia of the cornea and intracorneal lipophagocytic reactions occurred, but were quantitatively much less than those just described. It should be pointed out that these fractions, although clear, still contained much molecularly dispersed lipid.

Chylomicron-free, opalescent sera from dogs fed cholesterol in olive oil was also injected into a series of dogs' and rabbits' corneas. These animals had received 15-30 gms. of cholesterol dissolved in olive oil daily for two to four weeks, together with 0.5 lb. of horse meat. Their total serum cholesterols had shifted from the control range, 65-125 mg.%, to the 250-350 mg.% level. As with similar sera from cholesterol-fed rabbits, lipid was deposited intracorneally in diffuse form and led to lipophagic reactions within two weeks. Such reactions were intensified by injection of concentrated opalescent fractions obtained by high speed centrifugation. Injections of the clear subnatant fractions from such spins resulted in mild diffuse corneal sudanophilia in the zones of injection with little other reaction.

Lactescent human plasma from blood bank sources or postprandial serum from healthy adult donors was obtained. The lipid content of these ma-

terials was not unusual for nonfasting bloods. Microscopically visible particles were removed by low speed spins as described for animal sera. The residual opalescence of the centrifuged sera or plasmas varied, but was often prominent. The opalescence was not always cleared *in vitro* by heparinized dog plasma. Injection of the opalescent human serum or plasma intracorneally in dogs or rabbits gave rise without exception to diffuse stromal sudanophilia, but without lipophage activity. Concentration of the opalescent material by centrifugation at 30,000 r.p.m. for one hour resulted in the same intracorneal lipid deposits and lipophagocytic reactions that had been observed following the injection of similarly prepared fractions of sera of cholesterol-fed rabbits or dogs. A sample of concentrated opalescent human plasma that gave rise to lipophage reactions contained: total cholesterol 425.0 mg.%, free cholesterol 200 mg.%, lipid phosphorus 23 mg.% and fatty acids 84 mEq./1. Clear subnatants of human serum or plasma, centrifuged at 30,000 r.p.m. for one hour led to milder corneal sudanophilia without lipophage reaction. The sudanophilia, over a four- to six-week period, tended to fade and disappear. If such clear human serum fractions were injected two or three times into the same animal's cornea at four- to five-day intervals, the stromal sudanophilia was intensified, and scattered lipophages appeared. A similar intensification of corneal sudanophilia was encountered following injection of a centrifuged clear serum from a patient with familial hypercholesterolemia (cholesterol 600 mg.%). It should be pointed out that the sudanophilia invariably observed after the injection of human serum persisted long after microscopic edema resulting from the injection had disappeared.

In summary it can be said that clear control dog or rabbit serum from animals on low fat diets did not leave stainable lipids within the cornea following injection. Chylomicron-free, lipoprotein-containing fractions of sera from cholesterol-fed dogs and rabbits did result in corneal sudanophilia and lipophage reactions, especially when these opalescent fractions were concentrated either through the inherent level of the blood lipids achieved by feeding, or through centrifugation. Clear serum fractions containing residual lipoproteins produced less intense but definite corneal reactions. Single injections of chylomicron-free, clear or slightly opalescent, unconcentrated human serum or plasma from healthy donors with lipid levels in the usual range led to corneal stromal sudanophilia, but not ordinarily to collagen damage or to lipophage accumulation. However, injection of a fraction containing the concentrated materials responsible for opalescence of human serum led to intracorneal sudanophilia and to cellular reactions entirely

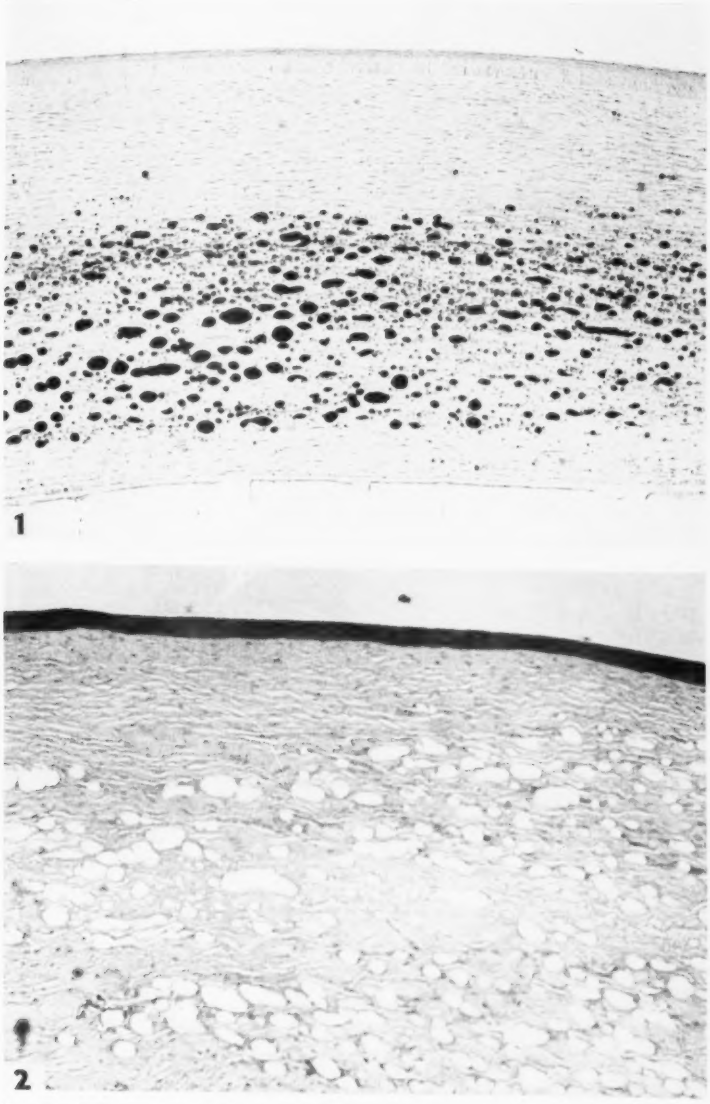


FIG. 1. Dog cornea fourteen days after injection of viosterol in corn oil. Sudan IV stain. Dark droplets of lipid remain unchanged. x150.

FIG. 2. Dog cornea fourteen days after injection of viosterol in corn oil. Epithelium above. Clear spaces represent lipid droplets. Fusion of collagenous lamellae in center with loss of nuclei. H and E stain, x200.

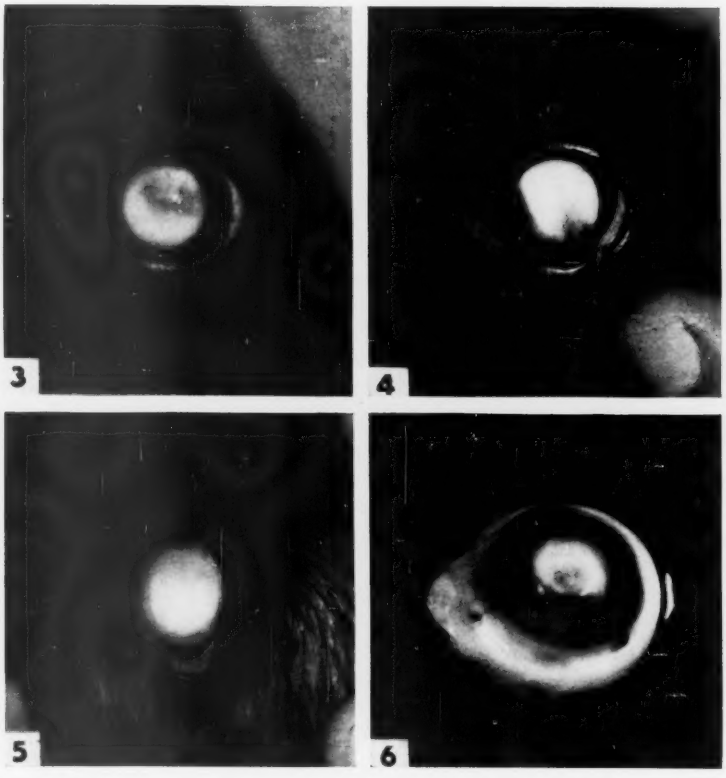
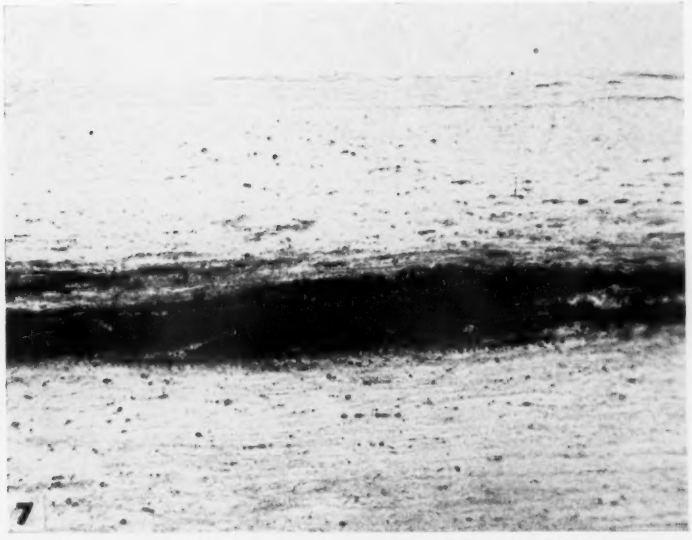


FIG. 3. Dog cornea six weeks after intracorneal injection of chylomicrons from dog serum. Cornea not vascularized.

FIG. 4. Dog cornea with central chylomicron-lipid pool six weeks after injection. Vascularization has begun. In zones of vascularization lipid is disappearing.

FIG. 5. Dog cornea three weeks after injection of dog chylomicrons. A small, round, edematous bleb is forming near edge of lipid pool where vascularization is occurring.

FIG. 6. Same cornea as in Figure 5 one week later. Corneal hemorrhage and ulceration have occurred.



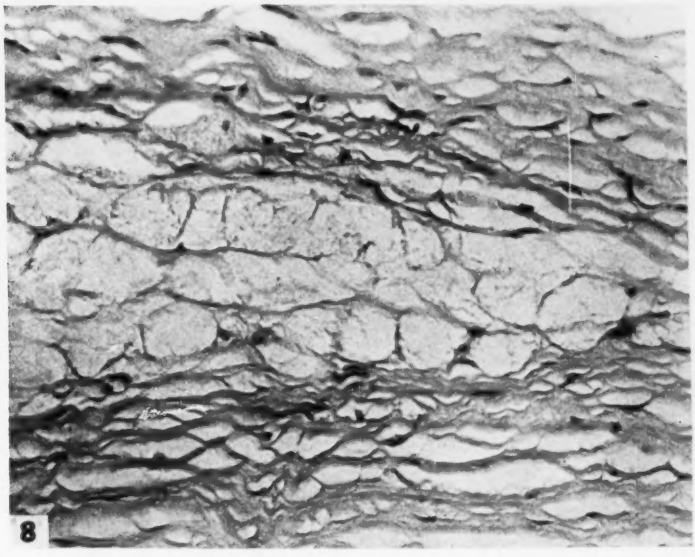
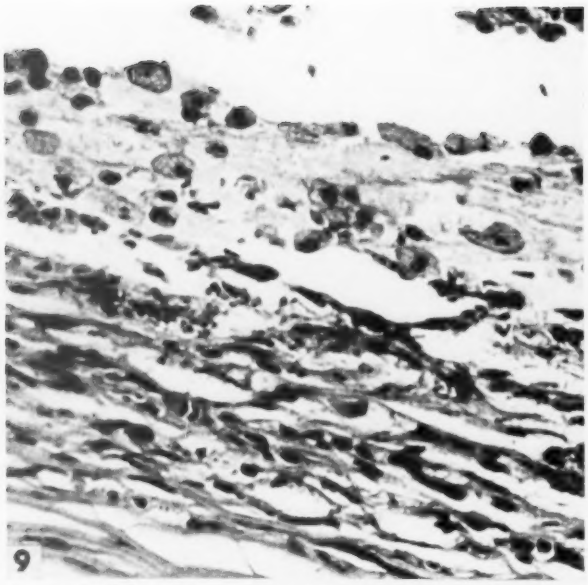


FIG. 7. Dog cornea twelve days after injection of dog chylomicrons. Sudan IV stain. Dark masses represent agglomerated chylomicrons. There is but little lipophagocytosis. x200.

FIG. 8. Dog cornea three months after injection of dog chylomicrons. Corneal fibers are spread by the lipid pool. Almost no cellular inflammatory reaction. No vascularization or fibrosis. H and E stain. x600.



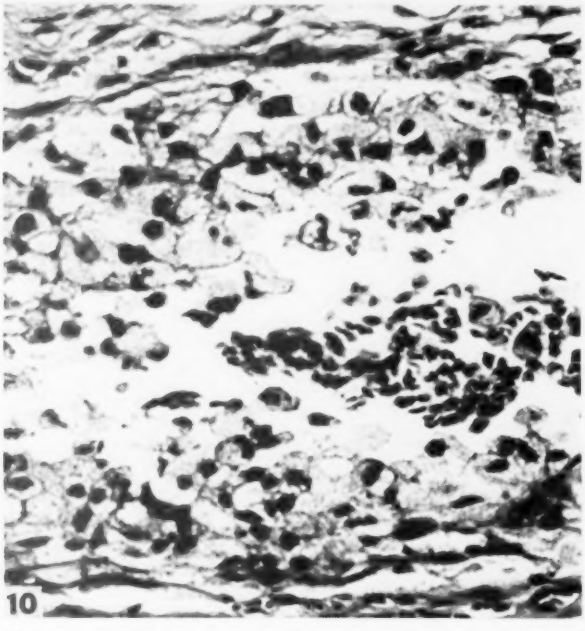


FIG. 9. Dog cornea thirty-five days after injection of dog chylomicrons. High power view of edge of lipid pool. Vascularization has occurred. Above, fused collagenous stroma with many lipophages. Below, vascular and fibrous pannus. H and E stain, x600.

FIG. 10. Dog cornea thirty-five days after injection of dog chylomicrons. There is hemorrhage in the center of the lipid pool and xanthomatous transformation of the vascularized lesion. H and E stain, x600.

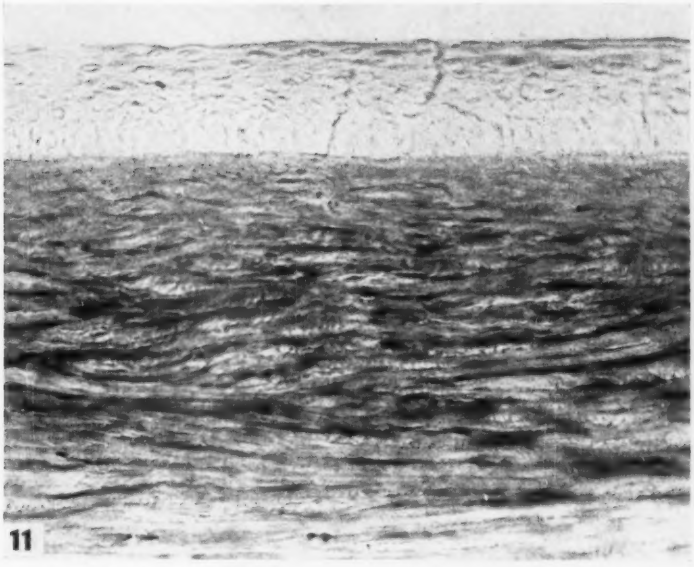
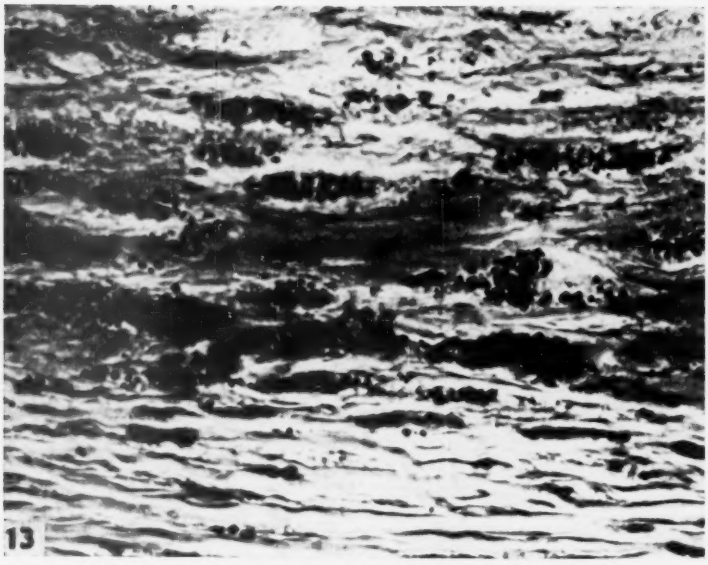


FIG. 11. Dog cornea ten days after injection of clear human serum. Sudan IV stain. Collagenous lamellae are diffusely stained with dark sudanophilic material. There is no edema and little lipophagocytosis. x440.

FIG. 12. Dog cornea ten days after injection of 0.1 ml. of clear human plasma. Sudan IV stain. Upper portion of corneal stroma is diffusely stained by dark lipoid infiltrate. There is no edema, and no fibrin deposit. x200.



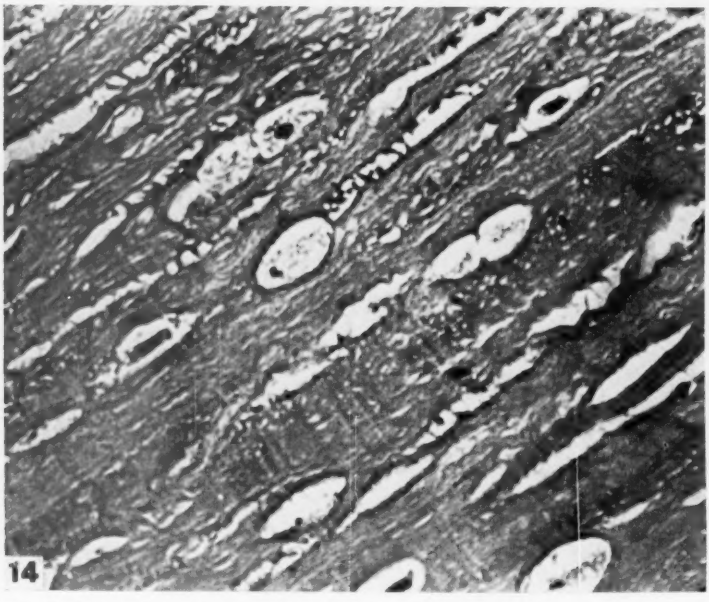
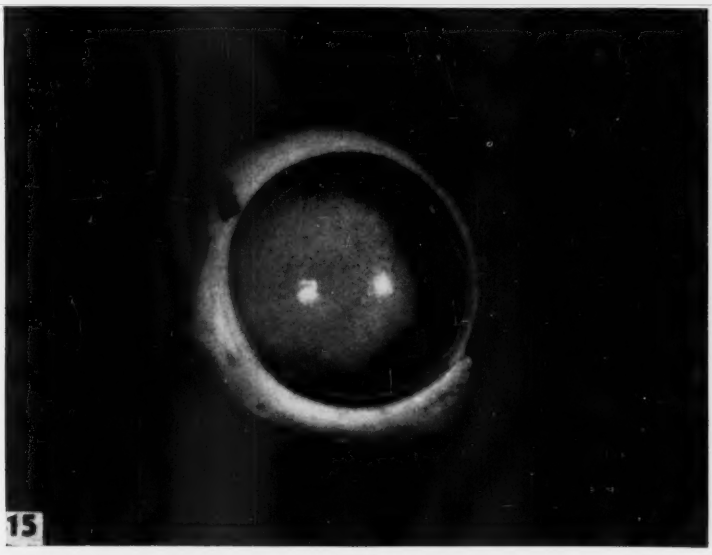


FIG. 13. Dog cornea fourteen days after injection of chylomicron-free, opalescent concentrate of human serum. Sudan IV stain. Near center swollen stromal lamellae stain diffusely sudanophilic. There is intense lipophagocytosis. x600.

FIG. 14. Same cornea as in Figure 13. H and E stain, x600. Fusion of corneal lamellae is well seen, with scattered foam cells.



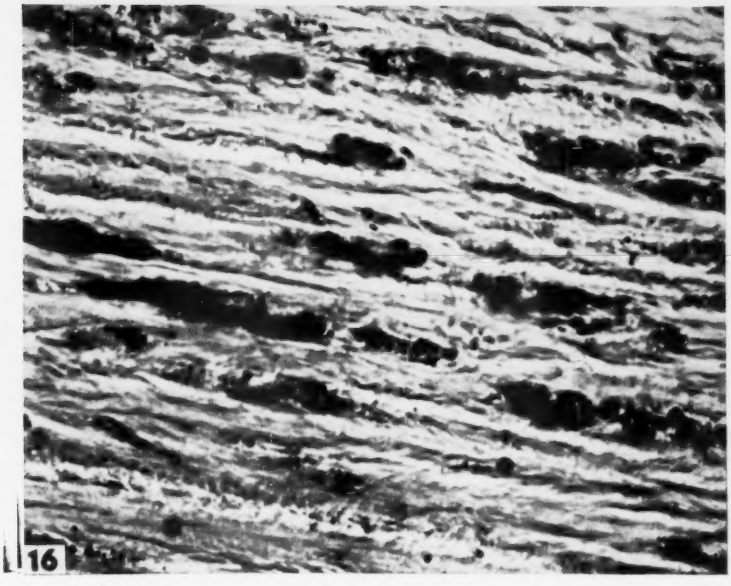
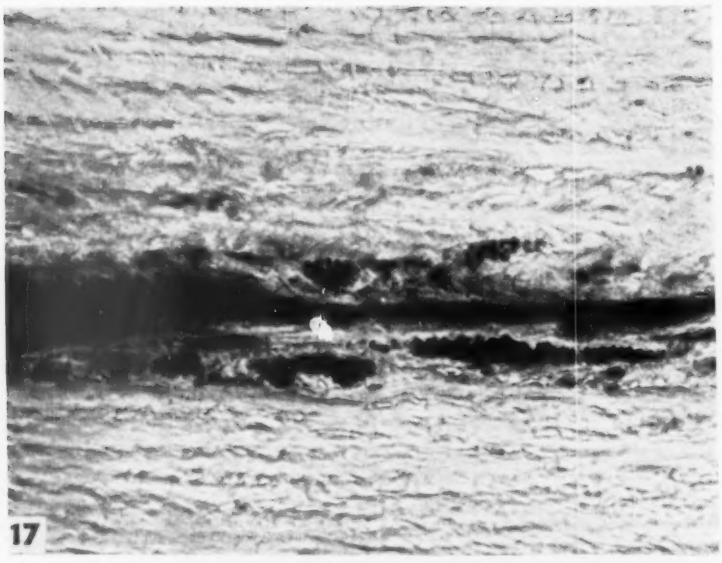


FIG. 15. Rabbit cornea seventeen days after injection of chylomicron-free, slightly opalescent serum from cholesterol-fed rabbit. A large corneal opacity has developed exactly in the lipid-infiltrated zone. There is no edema.

FIG. 16. Photomicrograph of the opaque corneal zone in Figure 15. Sudan IV stain, x600. Almost all dark sudanophilic material is within spindle-cell and large mononuclear phagocytes.



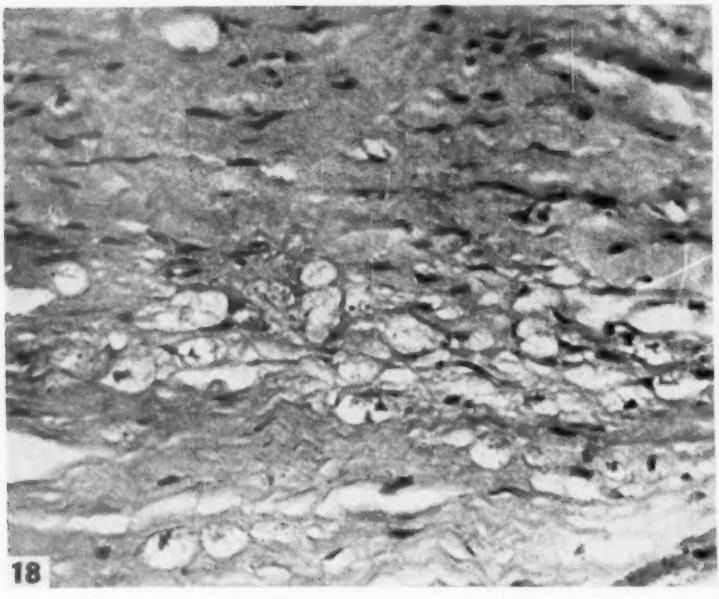


FIG. 17. Dog cornea, nine days after injection of denatured lipoprotein from clear human serum. Sudan IV stain, x500. Active lipophagocytosis.

FIG. 18. Same cornea as in Figure 17. H and E stain, x500. Stromal foam-cell granuloma, without vascularization or inflammatory cellular exudate.

comparable to those occurring after injection of unconcentrated sera or concentrated serum fractions from animals on cholesterol-rich diets.

The ultracentrifugally separable fractions of sera from cholesterol-fed rabbits and dogs and from humans responsible for these corneal reactions are the subject of continuing investigations. So far a few observations can be reported on known concentrated serum lipoprotein fractions. These were kindly provided by Dr. Lena Lewis, Dr. A. Scanu, and Dr. Irvine Page of the Cleveland Clinic. β lipoprotein and α-β lipoprotein concentrates (specific gravity 1.063, NaCl) with lipid content about five times that of the original sera were injected into a series of dogs' corneas, after dialysis against saline and the removal of some insoluble material. The lipoprotein solutions were faintly opalescent. Diffuse intracorneal sudanophilia resulted, with lipophage reactions similar to those occurring after the injections of sera from cholesterol-fed animals. A strongly opalescent concentrate of S_f 3-8 serum lipoproteins from human serum has led to intense sudanophilia of rabbits' and dogs' corneas, and after 9 days to active phagocytosis of lipid and to damaged stromal collagenous structures.

CORNEAL REACTIONS TO THE INJECTION OF INSOLUBLE SERUM LIPOPROTEINS

Throughout the experiments with serum or plasma injections intracorneally, it had been noted that lipid of lipoprotein origin was left in the cornea after corneal edema had subsided. This suggested that lipoproteins of serum might be rendered insoluble and that their lipids were retained in the cornea. The following further investigation was carried out: Chylomicron-free clear human serum from individuals with usual blood lipid values was dried under reduced pressure *in vitro* from the liquid state. As concentration proceeded, the serum was noted to become cloudy. After drying, the serum was reconstituted to volume with distilled water. The resultant solution contained large numbers of lipid-rich, insoluble particles. A sample of this material centrifuged off as a supernatant creamy layer and analyzed* contained: Cholesterol ester 20.1 per cent, free cholesterol 5.7 per cent, fatty acids 35.5 per cent, lipid phosphorus 11.1 per cent, protein 21.0 per cent. (Loss of lipid in the procedure was estimated at 6.6 per cent.)

Varying degrees of denaturation of lipid-rich serum protein in individual human sera occurred after standard drying. Such denaturation did not occur in serum from rabbits or dogs on low-fat laboratory diets. However, after short periods (two to four weeks) of cholesterol-fat feeding, when

* Through the kindness of Dr. Joseph Amenta.

serum cholesterol levels in these animals had risen to the 250-350 mg.% range or above, large quantities of the denaturable lipid-rich material were demonstrable in the sera. The quantity present seemed roughly proportional to the serum cholesterol levels and to the original opalescence of the serum after removal of microscopically visible particles.

Insoluble lipid-rich protein particles were prepared by rapid dehydration of chylomicron-free human serum or the sera of cholesterol-fed dogs or rabbits. These particles, after washing, were suspended in 0.9 per cent saline and were injected intracorneally into a large series of dogs and rabbits. The histologically observable events were the same whether the source of the material was human serum or cholesterol-fed dog or rabbit serum. The eyes cleared rapidly from the initial edema and opacity associated with the trauma and volume of the injection. The particulate masses could be observed grossly, lying in the corneal stroma. Histologically, after seven days these areas stained intensely with sudan dyes, and active lipophagocytosis at the poles of the lipid-infiltrated zones was occurring (Fig. 17). This lipophagocytosis became progressively greater and converted the infiltrated zone into a xanthomatous granuloma locally long before vascularization from the limbus occurred (Fig. 18). The foam-cellular areas observed gave strong Schultz tests for sterols. No foreign-body type giant cells were observed. If vascularization of the area took place, the lipophage activity already present was fortified. It should be emphasized, however, that local lipophage activity with these insoluble serum protein derivatives was prompt and vigorous. Polymorphonuclear leucocytic infiltration was not observed at any stage in the corneas. The corneal lesions following injection of *in vitro* denatured lipoproteins differed in no way from those following lipoprotein-rich fractions of animal or human serum.

CORNEAL REACTIONS TO INTRACORNEALLY INJECTED PLASMA FROM RENAL-ARTERY-LIGATED DOGS

In an effort to circumvent the possible disadvantage of utilizing heterologous lipid-rich plasmas (from man or rabbit) for observations of corneal connective tissue reactions in dogs, alternate plasma preparations were sought. It is known that loss of renal excretory function in dogs results in rapid elevation of the plasma lipid values.[7] In all, bilateral renal artery ligations were performed on 12 dogs. Their plasmas were collected after 72-96 hours without anticoagulant for injection intracorneally into other dogs. Plasma lipid and nonprotein nitrogen values for a representative sample follow: Total cholesterol: 386 mg.%, free cholesterol 112.0 mg.%, fatty acids 29.0 mEq./l., lipid phosphorus 15.5 mg.%, non-protein nitrogen 240.5

mg.%. The plasmas were centrifuged free of particles in cold paraffinized tubes. *In vitro* clotting times of these plasmas were markedly prolonged in comparison to those of control plasmas similarly prepared. They were injected into the corneas of anesthetized dogs in 0.1 ml. quantities. Without exception (12 corneas) intracorneal clotting of injected plasma from the renal-artery-ligated dogs occurred. It should be emphasized that these plasmas paradoxically exhibited prolonged *in vitro* clotting times. As has been mentioned, intracorneal clotting *has not* been observed with control plasmas from normal humans or dogs.

Masses of amorphous material having the histological appearance of and giving the staining reactions of fibrin were found microscopically at seven days in the corneas injected with azotemic plasmas from renal-artery-ligated dogs. In addition, the intracorneal fibrinous masses at this time interval were diffusely sudanophilic. Subsequently, at the 14-day interval, local organization of the fibrin was occurring at the margins of the deposited masses. Fibroblasts and mononuclear cells exhibited marked lipophagocytosis so that the investing connective tissue assumed a xanthomatous, foam-cellular appearance. In the fat stains, where lipid-laden macrophages were present, the diffusely sudanophilic fibrin masses had disappeared. The conclusion was drawn that large quantities of lipid had been deposited with the fibrin and that this was being phagocytized as a part of the local cellular reaction to the retained fibrin-lipid complex.

DISCUSSION

Corneal reactions to injected plasma, serum, and their contained lipids find certain parallels in the morphology of arteriosclerosis. In the cornea, the anatomical groundwork for pathologic reactions is similar to that in the intima of large arteries of man. Both tissues are avascular in that they do not have microscopically recognizable capillaries. Both the cornea and the arterial intima are rich in mucopolysaccharides and in collagenous connective tissue in lamellar arrangement. Both have in proximity a capillary blood vascular supply—in the cornea the capillary networks at the limbus, and in the artery the adventitial and medial *vasa vasorum*. In both, these capillaries are capable of rapid proliferation in support of ingrowing connective tissue. The chief anatomical and physiological dissimilarities are obvious. In the cornea there is no ambient blood flow, under pulsating intra-arterial pressure. Endothelium of course is also absent in the model. In the experiments reported, these deficiences have been arbitrarily circumvented by placing lipids, or serum or plasma containing lipids, directly into the cornea. This procedure, which introduces factors of trauma as well as

the substances under study, is perhaps justified by experimental evidence previously obtained in this laboratory that lipids present in the blood localize selectively at sites in the vessel wall where injury has occurred.

As a framework for discussion of the implications of the present observations for the pathogenesis of fatty arteriosclerosis, some of the similarities of the experimental lesions observed and those of the disease in man may be pointed out.

Lipid stains of small, superficial lesions of arteriosclerosis in young individuals, and therefore presumably early lesions, reveal the majority of the lipid to be outside of recognizable phagocytes, staining the connective tissue lamellae diffusely. Lipophage activity at this stage is limited to scattered spindle or large mononuclear cells present in the area. Injections of unconcentrated, chylomicron-free human serum or plasma into dogs' or rabbits' corneas leads to a diffuse sudanophilia of the corneal stromal lamellae similar to that occurring in arteriosclerotic lesions in man. Strongly opalescent, unconcentrated, chylomicron-free rabbit or dog serum or plasma after cholesterol-feeding greatly enhances corneal sudanophilia and lipophage reactions so that single injections of these substances often lead to gross opacification of the cornea. Even more intense reactions result from the injection of centrifugally concentrated opalescent fractions of human serum or of concentrated serum fractions from cholesterol-fed animals. Diffuse lipid impregnation of corneal stroma and foam-cellular accumulation before vascularization are thus especially associated with the presence of serum-lipoprotein lipids, and the concentration of these lipids in the cornea is of first importance in determining the intensity of reaction. After vascularization, either chylomicrons or lipids derived from lipoproteins contribute to extensive foam-cellular accumulations in the cornea.

In arteriosclerosis in man one of the most consistent findings is morphologically altered connective-tissue lamellae in the intima or inner media or in the connective tissue of plaques. These lamellae appear swollen, pale, "mucoid" in paraffin sections and with sudan dyes are stained diffusely or with lipids in very fine droplet form. This connective tissue change may occur in small, superficial lesions just under the endothelium, or it may occur in any part of the diffusely thickened intima or focally in the depths of a large intimal plaque. In many instances the pale swollen fibers can be observed to be disintegrating among the more compact, resistant elastic membranes. This disintegration is associated with accumulations of mononuclear cells with lipophage activity. The lipid-connective tissue complex is phagocytized, and the diffuse sudanophilia of the areas is cleared, the only lipid remaining being now within the aforementioned macrophages. Exactly

the same phenomenon occurs in the serum-injected cornea. Wherever damage to lipid-stained lamellae has occurred, diffuse sudanophilia is lost and the lamellae are replaced by lipid-laden macrophages. Further studies are being carried out to determine if deposition of lipid in itself is responsible for the collagen changes observed. The quantitative aspects of this subject may be of the utmost importance. In summary, it appears that lipoprotein-lipid of serum retained in avascular, connective-tissue sites, is phagocytized along with damaged connective tissue to result in a foam-cellular lesion, in many ways comparable to that of arteriosclerosis in man.

The incomplete inflammatory cellular reaction of small arteriosclerotic lesions of man is almost exactly duplicated in the corneal reactions to injected chylomicrons or to lipoprotein-containing fractions of plasma. Fixed tissue cells or wandering mononuclear cells alone are mobilized in varying amounts by the presence of these substances in the avascular corneal lamellae. The reaction to lipoprotein lipid is much more vigorous than to chylomicrons. With vascularization, the reaction to either chylomicrons or to lipoproteins becomes more intense with great increase in cellular exudate, which is for the most part mononuclear in character. The changes now are more like those seen in late arteriosclerosis in man. Boyd,[3] as early as 1929, emphasized the inflammatory character of certain stages of late lesions of arteriosclerosis. Certainly, cellular exudate, hemorrhage, xanthomatous transformation and scarring are commonly associated with vascularization of atheromatous accumulations in arteries.[17] It is tempting, on the basis of the observation that corneal ulceration occurs frequently after vascularization of the lipid-pool site, to suggest that, similarly, vascularization of superficial arteriosclerotic plaques may hasten ulceration and thrombosis by intensifying, after long relative dormancy, the local intramural inflammatory process.

Apart from the anatomical comparisons cited, the observations indicate that corneal tissue retains not only chylomicrons from injected plasma or serum, but also that it retains the dispersed lipids from chylomicron-free sera of normal humans. While no stainable lipids from sera of dogs or rabbits on low-fat diets are retained, large quantities are retained from the sera of individuals of these species after short periods of cholesterol feeding. The suggestion is inescapable that cholesterol feeding results in the presence in the serum of dogs and rabbits of a lipid-protein complex which behaves in the cornea as does a lipid-containing component of human serum. This material from animal or human source can be rendered insoluble by *in vitro* drying of serum from the liquid state. Such denatured material placed intracorneally gives rise to local lipophage accumulations. Whether dehydration

of lipoglobulins is the mechanism by which corneal connective tissue retains originally molecularly dispersed plasma lipids, or whether it is by simple mechanical filtration, or by yet another process, is the subject of further investigation.

Of interest was the often repeated observation that native human or dog plasma without anticoagulant did not clot within the cornea. On the other hand, plasma from azotemic dogs invariably clotted on injection. The reasons for these phenomena are not apparent and underscore how little is known concerning the precise conditions associated with extravascular clotting *in vivo*. Much diffuse lipid, presumably of lipoprotein source, was present in the intracorneal clots resulting from the injection of azotemic, lipid-rich plasma. In this connection Morrison[10] has shown that fibrinogen coagulating *in vitro* in the presence of serum carries down with it, or "occludes," large quantities of serum lipoproteins.

SUMMARY

Following prior observations that plasma lipids localize selectively from the blood at sites of experimentally produced arterial injury, an attempt has been made to study directly the *in vivo* reactions of avascular connective tissue to injected plasma, serum, and their contained lipids. A large series of rabbit and dog corneas injected *in vivo* with serum or plasma, or fractions of serum or plasma, have been observed from this point of view.

The normal dog or rabbit cornea contains no stainable lipid. The injection of physiological saline or of control, centrifuged plasmas or sera from dogs or rabbits on low-fat diets leads to no deposits of lipids within the dog's or the rabbit's cornea and to no corneal reactions except along the needle tract.

The injection of chylomicrons or of chylomicron-rich plasma or serum from animal or human source leads to prolonged retention of the contained fatty particles. The presence of large masses of chylomicrons in the cornea is associated with damage to collagen and with an incomplete inflammatory reaction consisting of mild marginal lipophagocytosis. Injection of small numbers of chylomicrons in their native medium elicits little corneal reaction and the chylomicrons tend to disappear from the cornea after three to four weeks.

If vascularization of the lipid-infiltrated area—corneal "fatty plaque"—occurs, the inflammatory reaction is greatly enhanced, with edema, hemorrhage, xanthomatous transformation, scar formation, and often ulceration. The investing pannus progresses through a foam-cellular stage to dense

fibrous scar, nearly devoid of lipid. The morphological similarity of this sequence to that occurring in late arteriosclerosis is pointed out.

Chylomicron-free, opalescent sera, or serum fractions, from cholesterol-fed dogs or rabbits or from healthy adult humans, when injected into dogs' or rabbits' corneas lead to diffuse deposits of sudanophilic materials in the stroma. These serum fractions are rich in low density lipoproteins. Varying quantitatively with the materials injected, the deposited lipids are phagocytized *in situ* before vascularization of the cornea occurs. Changes in corneal collagenous structures, the accumulation of lipophages and of fibroblast-like spindle cells may lead to progressive plaque-like opacification of the cornea. Attention is called to similar incomplete inflammatory reactions and morphological sequences occurring in nonvascularized arteriosclerotic lesions in man.

Drying of sera from normal humans or from cholesterol-fed dogs or rabbits renders variable quantities of lipid-containing proteins insoluble. This denaturable material is not present in control sera of dogs or rabbits on low-fat, low-cholesterol diets. It is present to some degree in all human sera so far tested. In insoluble form it elicits a prompt and vigorous lipophage reaction when placed intracorneally. This reaction is similar to that following the intracorneal injection of serum fractions, rich in molecularly dispersed lipoproteins.

In the course of the experiments, it has been observed that native human or dog plasma does not clot intracorneally following injection, whereas plasma from azotemic dogs coagulates massively in this situation. The intracorneal clots from plasma of azotemic dogs contain much stainable lipid.

Finally, studies are continuing on corneal connective tissue reactions to varying concentrations of more specific lipid-containing components of human plasma, and the relations of these components to diet—with the objective of learning basic facts necessary for the eventual modification of such interactions, which find their counterparts in arteriosclerosis in man.

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RADIATION AND THE HEALTH OFFICER

One hundred and ten years ago Edwin Chadwick began service on the General Board of Health in England. The creation of this agency marked the most important socio-sanitary step of the early nineteenth century. Just as Chadwick set the pace for that period, so Professor Winslow, in the twentieth century, with public health problems even more interwoven with the fabric of society, rekindled this torch of leadership.

It is perhaps inevitable that any one conscious of the depth and breadth of Winslow's vision would choose for the subject of this address an area of public health activity appropriate to that vision. He might himself have selected it for passionate leadership well in advance of the army of health officers. Because of the many opportunities for the prevention of disease, he would have sensed the impact of radiation upon society. The challenge of the new was always his hallmark and the subject would have been his. My thesis honors his name, while the lectureship gives me the opportunity to pay permanent tribute to a great teacher.

The United Nations Scientific Committee on the Effects of Atomic Radiation points out in 1958 that "living beings have always been exposed to ionizing radiation from various natural sources."[1] It may seem surprising, therefore, that such general interest has now been aroused in radiation exposure as a potential disease producer of increasing significance. The discovery of x-rays in 1895 and of radioactivity in uranium salts in 1896 had already demonstrated the eternal necessity for preserving an equilibrium between the benefits from scientific discoveries and the hazards associated with their application. Almost no scientific or technological advance has ever produced completely beneficent results without posing considerations of control of their adverse effects.

The prime advantages resulting to society in the development and use of x-rays in medicine and in industry cannot be overestimated. Their diagnostic, therapeutic, and research contributions loom large in the history of

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** The C.-E. A. Winslow Lecture, Yale University, November 20, 1958.

science. Their uses, however, led to the responsibility of voluntary and official agencies for delineating the limits of radiation to which the individual might be exposed with minimum risk.

To these developments of the twentieth century, tremendous man-made radioactivity has now been added. Its amount is unprecedented in all of history. The discovery of nuclear fission in 1939 and its rapidly emerging applications have given new emphases to the significance of radiation as a source of disease production. Wholly aside from the military implications of nuclear fission, the peace-time application of atomic energy and the prospect of similar applications with fusion processes have re-focused attention upon radiation hazards and their disease potential. The dramatic current emphasis, of course, has been upon the contamination of the environment by the explosion of nuclear weapons. There is general agreement, however, most recently confirmed by the United Nations Scientific Committee, that at the present rate of detonations these contaminations are far outweighed by the natural sources of radioactivity, by the potential hazards in the discharge of radioactive wastes from nuclear reactors, by possible accidents in reactors, and from the increasing use of x-rays, and of radioisotopes for medical and industrial purposes.

The radiations to which human beings are exposed from natural and man-made sources are similar in their physical nature and in general in the character of their biological effects. Unfortunately the amount of information with respect to the biological effects is far less than is the knowledge of the physics of radiation itself. The gross features of biological impact and the detailed quantitative aspects of their relationships to radiation dosage require much clarification.

At this moment no cure is at hand for the results of radiation exposure on man. The day may come when treatment of radiation sickness or of radiation effects in general may be available. Until that time arrives, both somatic and genetic effects lend themselves to control only via the route of prevention. This is singularly, therefore, a problem subject to the procedures generally used in the field of preventive medicine and public health. Unless biological effects are prevented through increasingly intelligent and perhaps even rigid regulatory measures, larger numbers of individuals will probably sustain significant injury.

It is a tribute to the atomic energy industry in this country, in England, in France, and in other European countries that the disabilities so far experienced in the rapidly developing atomic energy industry have been minor in their effect both upon the industrial worker and the population at large.

This experience, however, serves equally well as an indicator of the necessity of highly experienced supervision and of rapidly extending control over design, construction, and operation.

THE PROBLEM IN RADIATION

A few basic physical concepts are necessary for some understanding of the problem with which the industry is and will be concerned. The radiations include x-rays, neutrons, protons, cosmic rays, and the alpha, beta, and gamma rays from radioactive materials in general. The energy transfer from all of these radiations is similar in nature and all produce biological effects. In each instance the energy absorbed in the tissue determines the nature and the amount of the biological effect.

The lifetime of a given radioisotope varies with the nature of the material and unfortunately over short and extremely long periods of time. The familiar quantitative statement of such variations, namely, the half-life of radioactive isotopes, ranges from thousand millions of years (uranium 238) down to a very small fraction of a second (radium C').

The biological effects stemming from exposure to ionizing radiation differ in essential characteristics from almost any other biological phenomena with which we have hitherto dealt. Some parallelism appears to exist between the aging process in man and the biological effects of radiation. The aging process in fact may have some dominant consequences of radiation exposure. Aside from this comparison, however, the complexity of biological effects is not so easily matched in most of the preventable diseases with which the public health officer has been familiar. The type of radiation, its energy, the size of the dose, its distribution in time, its distribution with respect to the body or parts thereof, and the origin of the radiation all influence the result. Similarly, a series of biological factors affect the sensitivity of a tissue or radiation.

When one turns to genetic effects, even more complicated phenomena arise, which make the necessity, as well as the nature, of the control most difficult.

The population of the world is now exposed to natural and man-made environmental contamination. The relative quantities of exposure to each of these categories are reasonably agreed upon in the findings of the United Nations Scientific Committee, the U. S. National Academy of Sciences, and the British Medical Research Council.

Our concern at this time is dominantly with the man-made sources. They include (a) medical uses of x-rays and radioactive materials, (b) industrial

and research uses of x-rays and radioactive materials, (c) other sources, such as luminous dials of watches, TV sets, and shoe-fitting fluoroscopes.

More important, however, in these man-made sources is the radioactive contamination of man's environment, other than those listed above, which result primarily from nuclear explosions or from radioactive wastes disposal or from accidents involving dispersion of radioactivity. The radiation doses from the last two sources are still small, but in the future they may become appreciable.

In general, it is not an unfair conclusion that all steps designed to minimize unnecessary irradiation of human populations will bring great benefits in human health. The translation of such a principle into action will necessarily include the avoidance of exposure from medical, industrial, or other procedures for the peaceful uses of nuclear processes. This principle rests upon the assumption that even the smallest amounts of radiation may cause deleterious genetic, and perhaps also somatic, effects. The problem confronting us, therefore, presents one of the most complicated regulatory issues, simply because both somatic and genetic effects of radiation are often long delayed in appearance. Logic would dictate, therefore, that exposures should be limited to the minimum consistent with other positive values to man. Permissible exposures may be changed as increased scientific evidence warrants recalculation of the balance of values.

That the problem has already reached significant proportions in our country may be illustrated by a number of markers of development in the nuclear fission field. In the period from August 1946 to December 31, 1957, over 104,000 shipments of radioactive isotopes had been made in the United States, with a total of 385,826 curies of activity. In the first six months of 1958, the shipments aggregated 6,278 with an activity of 70,359 curies. Such shipments are increasing with great rapidity. Some of these radioisotopes are of long half-life and of considerable importance to public health workers. For example, up to the present time, almost 400,000 curies of cobalt 60 have come into general use in this country and the amount of cesium 137 is climbing rapidly.

The number of nuclear reactors built, building, or planned in the United States, as of June 30, 1958, is now 318, of which 138 are already in operation and an additional 109 are being built. These cover high temperature reactors, low temperature reactors, and critical assembly facilities. All involve problems of site selection, of waste control (liquid, gaseous, and solid), and of precautions against the occurrence of accidents.

The possibilities of industrial, medical, and general research application are such that extension of use of sources of radiation will move forward

with great rapidity. At each step in this progress the responsibility of the health officer expands. Materials of potential hazards occur at every step in the evolution of this industry, from the initial mining of radioactive ores to the final use of radioisotopes, x-rays, and other sources of radiation.

Some examples of the nature of these problems may be helpful. They are by no means all-embracing in scope, but they are chosen to illustrate the kind of difficulty with which administrative agencies are and will be increasingly confronted.

EXAMPLES OF SOURCES OF RADIATION

Diagnostic radiology

The evidence so far accumulated suggests that genetic damage to humans due to exposure to ionizing radiation is greater than was previously assumed. Since the judicious use of diagnostic radiology has been one of medicine's most important tools for almost half a century, a great deal of emphasis is now being placed upon the actual exposures being received and the genetic consequences thereof. As to the former, detailed studies are now increasingly reported in the literature which provide quantitative evidence of the nature and the amount of the exposure in diagnostic radiology. The genetic consequences are under similar detailed scrutiny, with of course much more delayed detailed and total findings.

The investigations and the scrutiny of procedural methods already suggest broad significant modification in current radiological practice. The U. S. Public Health Service, followed by a number of the states, has already promulgated revisions in tuberculosis x-ray case-finding practice. Modifications in policy and practice in the use of x-rays during pregnancy have also resulted from this emphasis. General hospital practice has led to significant control of the indiscriminate use of the fluoroscope.

In the more advanced hospitals detailed supervision of both diagnostic and therapeutic use of radioisotopes have come into force. Somewhat more elaborate administrative control units have appeared throughout the country, with astonishingly detailed regulatory programs already operative.

All of these measures have taken as their basic assumption that the use of radiation in one form or another represents a fundamental technique bringing high fruits to society. All of the studies have disclosed opportunities for a more intelligent application of such fundamental techniques, while reducing materially the objectionable impact of radiation upon man. Coupled with these changes in practice have come systematic inspections of all equipment to insure that radiation exposure, not only of the general population, but of the instrument user is reduced to a minimum.

Chamberlain[5] has recently summarized the situation in the following terms: "The present exposure to radiation for medical and dental purposes can be appreciably reduced by techniques and methods that are already available. These are economically feasible, and largely require only interest, education, and the use of well established information and instruments." The quantitative significance of this conclusion is enhanced when it is recalled that somewhat over 130,000 roentgen ray machines are in use in the United States. One-half of these belong to dentists and hospitals and the other half to doctors, osteopaths, chiropodists, and chiropractors. The advances to be gained by the judicious use of such equipment should not be dissipated by undue exposures which bring only danger either to the general population or to the practitioner. In this field of activity, as in so many others of technological advance, all of our control history indicates that "we may have our pie and eat it too."

Accidents

The potential hazards arising from operation of nuclear reactors are very great. The possibility of damage from nuclear explosion in nuclear reactors is small, but the accidental release of fission product inventories accumulated in the operation of reactors could cause extensive damage and destruction. Numerous studies have been made of these potentials, all of which are carefully scrutinized within the Atomic Energy Commission review. The problems are highly complex, but the record up to the present time has been good.

It must be recalled, however, that a conceivable accident in a power plant of 400 to 500 megawatts could lead, under a pessimistic combination of circumstances, to the fatal exposure of three or four thousand people, injury to hundreds of thousands and contamination damage over thousands of square miles.

It becomes essential, therefore, that ample precaution and safeguards are taken to insure that dangerous releases of fission products do not occur, even from smaller plants where the potential hazards are materially less.

These factors affecting safety require a maximum of application of technical knowledge, a rigid adherence to design objectives and to an equally rigid control of operational practice. All of these in turn require thoughtful selection of sites, so that the consequences of either the unexpected or the improbable accidents are reduced to an absolute minimum. In every instance, reasonable assurance must be afforded that the facility can be constructed and operated at a proposed location without undue risk to the health and safety of the public. If such determination of criteria dominates

normal operation, the radioactivity released beyond the site boundary must remain below the maximum permissible levels for continuous exposures. In the case of accidents, even from the worst credible one and under the most adverse dispersion conditions in nature, the doses beyond the boundary of the site must likewise be below the permissible (once in a lifetime) emergency doses.

The site-reactor design complex must be considered not only from the viewpoint of the designer and operator, but also of those responsible for the health and safety of the community. The accidents at Windscale, England, in Canada, and in the United States, all having fortunately minimum consequences to the public health, do remind us that decisions of a public health nature will be continuously required in the future. Shall arable land contaminated in a nuclear accident be taken out of use? Shall contaminated milk be destroyed? Shall populations be evacuated? Shall contaminated vegetables be distributed to the market?

Simple adherence to maximum permissible levels of concentration of various radioisotopes in the air, in water, and on land do not offer a complete handbook basis for administrative action. In every instance such permissible limits will have to be tempered by administrative decision as to the balance of values to be obtained in embargoes versus biological effects on the human population. Such administrative decisions will become complex and burdensome. That they cannot be avoided is almost axiomatic. That they will be met through current administrative practices is a reasonable prospect.

The safety record of reactor operations in the United States of America has been exceedingly good, due to rigid management and safety precautions. That accidents have occurred and will occur in this industry, as in others, really emphasizes the fact that administrative practice will have to be geared to such occurrences, particularly in view of the fact that such accidents hitherto recorded have been due both to personnel and to mechanical failures.

Waste products

The nuclear fission industry, as every other industrial process, will be plagued by the production of gaseous, liquid, and solid wastes. The nature and amount of these wastes differ with the type of process and reactor used. The amount of radioactivity inherent in each of these sources is highly variable. The mixtures are complex, not only with respect to their radioactivity, but with respect to their physical and chemical characteristics.

The treatment, management, and disposal of these wastes is expensive and technologically difficult. With the exception of those wastes which are

low in radioactive constituents, virtually none of the wastes of high activity emanating from these industries are discharged to nature even after varying degrees of treatment. It is unfortunately true that no man-made equipment can destroy radiation. The only effective means is via the natural process of decay. Therefore, tanks are used for storage of such materials, even for as long as a quarter of a century. This method is not only expensive, but actually does not solve the problem. Good housekeeping is the key to safety. Such housekeeping rests upon the precepts that the amount of wastes should be kept to a minimum, should always be contained, and should be kept separate and distinct for identification and monitoring.

The familiar processes of the chemical industry are applied in this field with only reasonable satisfaction. Methods of precipitation, filtration, ion exchange, distillation, and biological processes have been used to reduce, to concentrate, and to make manageable the holding of these radioactive materials. Where these are released to nature, the permitted concentrations necessarily provide for a high factor of safety.

Concern for the air we breathe, the water we drink, and the food we eat makes the control of these sources of contamination one of the great challenges of the future. It is the only series of wastes in all of our industrial history which cannot be released to nature and for which only time for aging provides the safest answer. Even the location for the managed deposition and holding of these materials becomes a matter of interest and concern to society. Decision with respect to this and many other aspects of the developing industry cannot be left exclusively to the producer.

Transportation

Many solid, liquid, and gaseous substances of widely varying radioactivity are now shipped through a great many areas of the United States. Their hazards range from the so-called radium paint used on instrument dials, of very low activity, to materials of high activity and long half-life. The latter must be packaged and transported with extreme care to reduce the number and severity of accidents and to prevent either the liberation of radiation or the contamination of the environment.

Transportation has been given detailed attention by the Atomic Energy Commission with particular reference to type of container, freight, safety hazard, costs, transportation mode and vehicle, and route of shipment. Safe transportation of these materials along public transport lanes from production sites to consumer, and to processing or disposal sites involves many problems of health and safety. It is an area which is still under careful evaluation. The problem has been managed up to the present time with both

skill and absence of difficulty. The volume of high level liquid wastes will mount decade by decade and will reach millions of gallons by 1980. Each year, therefore, the complexity of the problem will increase correspondingly. Products of less radioactive significance now represent tens of thousands of shipments annually. The participation of official health agencies in the appraisal and control of these operations is virtually nonexistent.

PRESENT STATUS

A tentative summary of the radiation problem would lead one to the conclusion that sources of radiation are rapidly on the increase, that no effective method is available for preventing damage to a cell by ionizing radiation and that treatment, once damage has been done, is nonexistent. Only one recourse remains to society in the control of this important addition to scientific knowledge and use. Access of radiation to human beings should be prevented and controlled by every conceivable and ingenious means. The prevention of accidents in turn requires the highest degree of supervision of all sources of radiation and a continuing and perpetual alertness to the observance of evolving criteria and rules of operation and behavior. This task is already so complex that it offers a challenge hitherto quite unknown in public health control procedures, although it will parallel the more familiar ones in the historical interposition of barriers between disease and man.

ADMINISTRATIVE IMPLICATIONS

An attitude of precision of control must be bred into each designer, constructor, and operating staff in order to make sure that hazard potential has been reduced to a minimum and that precautions in case of unexpected accident will be intelligently implemented. The mere listing of the uses to which the modern world has put sources of radiation tends to confuse and to horrify the abstract and theoretical designer of regulatory procedures. History shows that administrative control has proceeded in many areas of complexity in modern living on an ad hoc improvisation basis. Supervision results from daily development of the details of problem control. Before long, regulatory procedures are in force and controls and counter-controls have been developed. Operating personnel pursue their tasks quite unconscious of all of the precautions which they skillfully practice. The general population goes about its normal daily business unaware of the checks, the restrictions, and the defenses which some agency somewhere has carefully devised and enforced for its protection.

The development of radiation use has been accompanied by rapid parallel understanding of hazards and of devices and practices for their prevention

and control. The consequences of accidents are increasingly understood, but experience with them on a large scale still remains to be developed. Only when such accidents have occurred will the extent and severity of their consequences be fully appreciated. The lessons to be learned from disaster may be anticipated in theory, but must actually be learned the hard way in reality.

The function which already confronts the health officer with respect to radiation is one which must give him pause, but not one which should lead him to avoid the responsibility which is primarily his. Within the hospital, industry, and general research areas, sources of radiation have mounted in almost astronomical fashion. Each hospital will ultimately have to provide a radiological control committee or board. The supervision of use and practice by such an overlying committee is undoubtedly irksome, but essential. Diagnostic and therapeutic uses of radiation are so great in every hospital that internal policing by a responsible unit is not only indicated for the future, but is increasingly practised in major institutions today.

An agency external to the hospital, such as a health department, assumes a responsibility for the final disposal of radioactive wastes from every medical institution. Such wastes range all the way from radioactive animal carcasses to materials discharged to the atmosphere and into the public sewers. Solid materials likewise require supervised packaging and ultimate placement in a safe permanent depository.

In industry,[4] powerful x-ray sets are now employed in open areas for taking pictures of welds in the plates of ships under construction or of large structures being erected or tested outside of the buildings. The list of other uses mounts, of which only a few are as follows: shoe-fitting fluoroscopes, static eliminators in textile and similar manufactures, isotopes for thickness gauges, examination of welds with cobalt 60 or other sources, measurement of solidification boundaries in castings, the emerging potential of sterilization of food and drugs, the activation of chemical processes, the uses of radioactive materials in agriculture, the attempt to pasteurize dairy products by radiation, the treatment of meats for the elimination of trichina, and the handling of hides for the destruction of anthrax.

The use of reactors for mobile equipment poses unexplored and at the moment unsolved national and international issues. The reactor in submarines, in surface ships, in air craft, and in space rocket propulsion—all inevitable consequences of modern science—create conditions of potential risk. All of them must be so designed, constructed, and operated that this risk is kept to an absolute minimum, with normal operation or in accident. State and national responsibility must be ultimately expanded to international control. No part of the world will remain free of the control necessi-

ties here discussed. Already some significant efforts and arrangements have been developed for international participation in appraisal of problem and in control of effects.

In the control and regulation of radiation sources, universal agreement must be found on the kind and magnitude of criteria which should be enforced. Fortunately, most individuals and public health agencies appear to be ready to accept the values hitherto made available by the National Committee on Radiation Protection. The Committee's deliberations since the 1920's have gained the respect of scientific workers. The maximum permissible levels which it and the International Committee on Radiation Protection have promulgated represent the bases of most state, national, and international applications. The mere existence of these permissible levels, however, is not a substitute for continuing administrative judgment. Each permissible level suggested implies a certain degree of risk. The health official in every instance of action is confronted with balancing risk against values to society. He must from time to time determine whether acceptance of the maximum permissible level or exceeding it is the part of regulatory wisdom. For example, should vegetables or milk be banned from use if they exceed the maximum permissible levels now at hand? The health officer cannot avoid providing a judgment as to when and at what level of concentration materials should be prohibited for wide distribution and consumption. It will be the part of wisdom in every significant decision to summon up the best advice in this complicated area of hazard. Multiple scientific disciplines are the bases of this industry. This practice of using advisory service is a common and familiar organizational asset.

Departments of labor, highway commissions, boards of economic control, health departments, and other agencies have responsibility in this rapidly evolving field. Past experience has shown that central responsibility for such hazards as are here under discussion has historically rested upon departments of health and of labor. In Government, the partition of such responsibility as between the workers in industry and the general public has been reasonably delineated and has been functionally successful.

It is doubtful indeed whether any new agencies are needed to encompass the broad role here described. It is equally doubtful whether the establishment of a coordinating office is indicated in most of the operations of our state agencies. Those less familiar with regulatory activities in the field of health and industrial safety visualize increasing problems of competitive action, variable standards for health and safety, and confusion in public regulation. Most of these fears have not been exemplified in the evaluation of health and safety controls in other fields historically affecting society.

There is little reason to suppose that such organizational confusion and competition will result in this area. Competition certainly can be avoided by placing this responsibility in the two primary agencies within the states in which precedent for prevention of disease has shown clear and successful performance.

EMERGING PUBLIC HEALTH RESPONSIBILITY

Workers in public health could not long ignore the issues here discussed without increasing consciousness of their responsibility to society. In different countries, different approaches to the dual areas of the developer and of the regulator of hazard have been manifested.

In the United States, the Atomic Energy Commission, certainly for the first ten years of its activities, accepted the contrasting obligations of developing both the science and art of nuclear fission operations and of assuring the health and safety of worker and general public. As works increased both in number and in complexity, this duality of function has become increasingly untenable, if not actually embarrassing.

From the beginning, the Commission, always aware to a high degree of its responsibility for health and safety, created a Committee of Review of all reactors to be built. The present Advisory Committee on Reactor Safeguards now serves both the Commission and the Joint Congressional Committee on Atomic Energy. The ACRS is now a statutory committee created by Congress.

As problems multiplied, however, the AEC took the additional step of creating a full-time Hazards Evaluation Branch as a separate entity from its units engaged in research and development. The division of Biology and Medicine, of long standing in the Commission, continues its function as an advisory unit and as a broad stimulator and sponsor of pertinent research.

In this evolving process over the last two decades, official public health agencies, both federal and state, remained largely on the periphery of action rather than in their rightful position of exercising responsibility in arriving at criteria of safety, of participation in the selection of sites, of taking decisive action on waste management, of reviewing the manifold decisions with respect to mobile reactors on land, air, and sea and the use of land and sea for deposition of radioactive materials.

Fortunately, this state of apathy or reluctance has changed rapidly. The U. S. Public Health Service has recently established a separate division to concern itself with these problems and has created a special advisory board to the Surgeon General for the continuing examination of radiation problems and of the position which the Service should assume in this area.

From August 1954 to July 1958 eight states have adopted broad, comprehensive radiation control regulations, e.g. California, Connecticut, Massachusetts, Michigan, New York, New Jersey, Pennsylvania, and Texas. Thirteen other states now require registration of hazardous radiation sources. Still more are concerning themselves with radioactive wastes so that their management and disposal will accord with those required for industrial wastes in general. Undoubtedly before long, every state will adopt some procedure for health and labor departments to assume these responsibilities.

Hilleboe and Rihm, Jr.,[8] have recently succinctly summarized the essential reasons for the movements briefly noted here, in the following terms:

"Atomic fission and fusion, spawned in this fabulous 20th century, have radically changed the environment for every living creature on the face of the earth. Only by preventive action now and continuing vigilance in the future, can we hope to avoid the devastating effects of unnecessary exposure to ionizing radiation."

In England, the evolution of control has paralleled, in essential principles, that in the United States. In recent years, however, the industry has been converted by parliamentary act into a quasi-independent agency having some of the characteristics of a publicly owned private company. In this form, its activities are at least in theory subject to the review and control of health, industry, and housing and planning public bodies.

The Windscale accident[6,7] perhaps focused attention on the inadequacies of the relations of the atomic energy industry to the orthodox regulatory groups, and in turn to the natural users, such as water purveyors. This unhappiness at the present state of affairs is best exemplified in the Interim Report to the Executive Committee of the British Water Works Association by its Subcommittee on Radioactive Substances.[8] The following excerpts are pertinent:

"The machinery of ad hoc administrative arrangements may well have been adequate in the early days of the development of the Atomic Energy Industry. These days have passed and there is now a clear need for the arrangements to be put on a proper footing and for the water authorities to have the right to be consulted and warned about the things which are likely to affect them and their duties in respect of the public water supplies of the country."

The Fleck Report[6,7] on the Windscale accident makes it abundantly clear that there are serious deficiencies in the machinery for dissemination of knowledge, and for consultation and cooperation between those responsible

for the atomic energy industry and those who may possibly be affected by its operations.

Knowing the flair of the English for resolving the tangled skeins of administrative competitive practice, it is predictable that these increasing evidences of lack of integration will be the subject before long of parliamentary inquiry and correction. The industry is obviously moving out of its infancy both in the United States and in England. Its maturity will bring it into the "wholeness" of supervision to which every other industry has long been exposed to protect the public at large. That supervision should be skillful, informed, and minimal consistent with health and safety.

INTERNATIONAL ASPECTS

Scientific knowledge in this age of communication speed has wings. No country long provides containment of knowledge. This is fantastically true in atomic energy. No country in the world is unaware of its potentials. Some countries look to it as their salvation, which it is rarely likely to be.

In consequence of these hopes, however, the values as well as the problems of nuclear fission and of radiation have leaped national boundaries and created national and international issues. By the early sixties power reactors producing over a million kilowatts will be operating in Europe, exclusive of England. Research and power reactors will go forward at the same time in some forty nations of the world. Centers for radioisotope research, schools for training of professional and subprofessional workers, and international distribution of sources of radiation have moved forward with such rapidity that even an assessment of their impact becomes wellnigh impossible.

Radioactive materials are moving on international routes, chemical processing of fuels will proceed in regional plants, discharges of low level wastes will reach international streams, the oceans are receiving and will continue to receive increasing amounts of radioactive liquids and solids, and locations of reactors on or near borders of nations will increase. Virtually all officials are searching for appraisal, understanding, and agreement between nations as to the management of this new material, process and effect. For the first time in history, the discharge of radioactive wastes into the seas found a place on the agenda of the International Conference on the Seas at the 1958 Geneva sessions.

In essence, every one is on a search of how to live with radiation. The temperament guiding most participants in these national as well as inter-

national deliberations was well phrased by Karl Z. Morgan of the Oak Ridge National Laboratories in these words: "One should not fear radiation but one must respect it!" Formal international agreements will ensue which will undoubtedly be based upon these precepts.

SUMMARY

The last twenty years have been the period of greatest progress in understanding and development of nuclear fission and fusion in the history of the world. This progress has been paralleled by unprecedented use of radiation sources for biological and industrial purposes. In these advances, major collateral progress has been made in the modification and creation of materials, processes, and equipment which would not otherwise have occurred for several decades to come.

The hazards resulting from these developments must be balanced against the obvious and great benefits of radiation uses. Although the latter are at times exaggerated by some of the enthusiastic supporters of this work, no one may fairly deny the fact that radiation use will produce great advantages to society. Ways must and will be found to make such use compatible with the health and safety of man.

The theoretical listing of the administrative measures thus required inevitably stress the multiplicity and complexity of such efforts. Society, however, has long met its environmental challenges by ad hoc improvisation of administrative structures and techniques. If paraded before us, they would stagger us by their large number and complexity. They are pragmatic results of necessity and they work. The control of radiation use will likewise fall into place in the structure of orthodox agencies long experienced in assuming new, while relaxing old, obligations.

Health departments are rapidly extending their responsibility in these areas.[1] They do so because radiation hazards are real, exposure is increasing and adds to body stresses, and because it can be controlled and reduced without losing the increasing benefits and values of such application. It is of grave public health concern to control the limits of eventual contamination of the environment of reactors. Since it is practically impossible to remove radioactive contamination once it occurs, the ounce of prevention is literally worth a ton of cure. Prevention of disease is the health officer's dominant responsibility in this new area as in the more familiar domains of the past. That he can meet this challenge, once he fully recognizes it, no one should doubt.

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EVENTS

MICROBIOLOGY SEMINAR

November, 1958

REVERSE PASSIVE CUTANEOUS ANAPHYLAXIS. By Zoltan Ovary, Department of Microbiology, Johns Hopkins University, Baltimore, Maryland.

Passive cutaneous anaphylaxis (PCA) is the reaction provoked in the skin of an animal, the guinea pig, by the intradermal injection of antibody followed after a suitable latent period by the intravenous injection of antigen and dye. The antigen-antibody reaction increases capillary permeability, permitting the dye to pass through the endothelium and stain the animal's skin. The intradermal injection of histamine produces the same increase of permeability. Therefore, it is held that the increase of capillary permeability in the guinea pig's skin is caused by histamine liberated during the antigen-antibody interaction.

An inverse relationship exists within certain limits between the amount of antibody injected intradermally and the latent period; however, it is impossible to obtain any reaction with very large amounts of antibody if the latent period is less than 15-20 minutes. It is postulated that the antibody becomes fixed to the endothelial cells during the latent period. This "fixation" may be a dynamic process; Jancsò demonstrated that gamma globulins are constantly moving in and out of the cell. It is thought that antibody—modified gamma globulin that can combine with a specific antigen—replaces some of the nonspecific "normal" gamma globulin of the cell, and that the antigen-antibody reaction liberating histamine occurs on or in the cell membrane.

Reverse anaphylaxis refers to the injection of antigen before antibody. It is impossible to obtain a reverse anaphylactic reaction with most antigens (such as bovine gamma globulin or polysaccharide antigens). However, when rabbit or human gamma globulin is used as the antigen, reverse anaphylaxis occurs. This illustrates the necessity of "fixation" of antigen or antibody to the cells of the host in order for a reaction to occur; only rabbit or human gamma globulins are "fixed."

It is impossible to induce PCA using horse, chicken, or sheep antibody, such as anti-egg albumin, but rabbit, guinea pig, or human antibodies will give PCA. It is postulated that this discrepancy is due to lack of fixation of horse antibody to the guinea pig's cells. If rabbit or human gamma globulin is used as the antigen, the corresponding guinea pig, horse, chicken, or sheep sera (with anti-rabbit or anti-human gamma globulin) will give reverse PCA reactions.

Normal horse gamma globulin or horse anti-Pneumococcus type III antibody does not inhibit the PCA reaction of rabbit anti-egg-albumin since horse antibody does not compete with rabbit antibody for the receptor sites of the guinea pig's endothelial cells. However, horse antibody (such as anti-egg albumin) is present at the injected site, since it can compete with rabbit anti-egg albumin for the antigen (egg albumin) if the latter is given at

threshold levels. Therefore, horse antibody is not fixed to "receptor sites" as it cannot provoke PCA. Even large amounts of horse antibody (fifty times the amount of rabbit antibody) diffuse from the area rapidly, while rabbit antibody is still present.

D. W. B.

NU SIGMA NU LECTURE

December 10, 1958

SODIUM AND THE CENTRAL NERVOUS SYSTEM. By Dr. Robert E. Cooke, Professor of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, Maryland.

The site of the selective permeability exhibited by the brain has been postulated by some to be the perivascular space of Virchow and Robin, which is the extension of the subarachnoid space around small cerebral vessels. Current thought, however, favors the concept of a "blood-brain barrier" determined by the composition and sealing effect of a pericapillary ground substance. Cerebral capillaries are composed of capillary endothelium and a basement membrane, and are surrounded by closely applied end-feet from glial cells, rather than by any sort of space. The ground substance may be high in sodium, because the sodium content of brain is high and somewhat resembles cartilage in composition, leading to the speculation that the brain may be rich in intercellular matrix.

Isotope dilution techniques have shown that heavy water (D_2O) exchanges in the brain rapidly and freely on a molecular basis and does not require secretion of the choroid plexuses. Sodium exchange is likewise through the capillaries as well as through the plexuses, but is quite slow, requiring 62 hours to reach equilibrium with the "sodium space." Anions like thiocyanate and bromide gain access to the brain through the choroid plexuses via the cerebrospinal fluid. The ratio of these ions to chloride in brain after intravenous injection mirrors the ratio in CSF and not that in plasma.

Lowering the extracellular sodium concentration, as in Yannet's experiments in which glucose and water were injected intraperitoneally, causes water to rush into the brain, leading to somnolence and ostensibly lowered electroshock threshold. Acute hypernatremia, as in severe dehydration in small infants, has been shown to give rise to vascular damage (subdural hemorrhage), convulsions, and frequently irreversible damage. The bleeding encountered here may be due to a tearing of bridging veins as water leaves the cells rapidly and engorges the perivascular space. If a solution of urea rather than of sodium chloride is administered, neurological signs abate as secondary movement of the freely diffusible solute into the cells aids in the osmotic readjustment.

An effect of the sodium ion on membrane potential has been postulated to explain its actions, but such a theory would necessarily predict, from the Hodgkin equation, that a rise in extracellular sodium would cause a lowered membrane potential. This leaves unexplained Woodbury's observation that saline drunk by adrenalectomized rats *raises* their electroshock threshold,

and lowering of extracellular sodium concentration lowers electroshock threshold. Actually the P_{Na} in the Hodgkin equation is low, and membrane potential would be changed little by changes in sodium, more by changes in chloride, and still more by changes in potassium concentration. It seems reasonable, therefore, to attribute neurological effects in hypernatremia to vascular injury. Clinically, patients do not have seizures until the sodium concentration is falling, which is consistent with the hypothesis that the elevated sodium keeps the seizure threshold up during the period in which vascular changes have predisposed to the seizure state. Once the sodium concentration has been lowered, the neurons may fire, and the seizure does in fact occur.

M. S. M.

ZOOLOGY JOURNAL CLUB

January 7, 1959

PHYSIOLOGY OF HIBERNATION. By Charles P. Lyman, Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts.

Hibernation occurs primarily in three orders of mammals, the Chiroptera, Insectivora, and Rodentia. The rodents as a group are the hibernators *par excellence*, and the present results deal exclusively with them. Examples are the ground squirrel, woodchuck, dormouse, and hamster.

All rodents curl up in hibernation as if to reduce heat loss, but the gradient is so small that it can hardly be significant. The body temperature follows the environmental temperature closely (usually about 1° C. higher) between about 2° C. and 17° C. Outside this range the animal usually wakens. Oxygen consumption is about 1/30 to 1/50 normal, breathing rate is one to two per min., and heart rate is one to twenty per min. (as opposed to several hundred per min. normal). Diastolic blood pressure may be reduced below 10 mm. Hg. Electrical activity in the cerebral cortex is reduced considerably; the hamster is extreme, showing no activity at all, spontaneous or induced. Contrary to most mammalian nerves, which show total blockage at 10° C., hamster nerves function to about 3° C. Similarly, the heart does not fibrillate until about 3° C. There does exist some mitotic activity and cell aging.

Conditions of homeostasis still obtain during hibernation. Blood sugar level and pH are regulated normally. CO_2 is regulated; there is a linear relation between per cent CO_2 and respiration rate, and the animal wakens if the CO_2 reaches 8 or 9 per cent. There exists some homiothermism to maintain the animal always slightly above the environmental temperature.

Waking from hibernation is characterized by an increase in oxygen consumption, heart rate, and blood pressure. The temperature increases anteriorly much faster than posteriorly due to a process of differential vasoconstriction. Muscular activity is increased, accompanied by much shivering. There is rapid blood glucose mobilization, with a concomitant depletion of liver and muscle glycogen.

Entrance into hibernation is characterized in some species by early preparation, such as food storage and fat accumulation. The body fat often

becomes less saturated such that its melting point is lowered. Oxygen consumption, heart rate, and blood pressure decrease before the body temperature falls, indicating that the temperature drop is probably the result of, not the cause of, the other phenomena. Rather than the normal process of vasoconstriction which occurs in the cold, temperature decrease upon entering hibernation brings about vasodilation.

F. M. W.

January 14, 1959

THE BIOCHEMICAL CYTOLOGY OF THE LIVER CELL. By Alex B. Novikoff, Albert Einstein Medical College, New York, New York.

In recent years the techniques of differential centrifugation, electron microscopy, and cytochemistry have added much to our knowledge of the cell. Information gained from these sources, when related to a multitude of pertinent biochemical data, provide the beginnings of a solution to the problem of relating structure and function in the living system.

Electron micrographic studies of histochemically treated rat liver tissue have yielded information on the precise localization of ATPase. The bulk of this enzyme is removed or inactivated during Formal-Calcium fixation and other histological procedures; it does, however, remain in cells associated with the bile canaliculi. Electron micrographs of histochemically treated tissue show that the ATPase is found in or on the plasma membrane, especially where the latter projects into the lumen of the canaliculus. The enzyme is similarly found at the surfaces of both endothelial glomerular cells and base cells of the proximal convoluted tubules of the rat kidney. In all of these cases it seems to be located where relatively high metabolic activity might be expected to occur at the cell surface.

These studies, and several others, emphasize the fact that the term "liver cell" is a great generalization. Because of such things as the differential distribution of many enzymes, and the differences in mitochondrial morphology of peripheral and central liver cells, a great heterogeneity of cell types exists. These facts re-emphasize the inseparability of structure and function in the living cell, and suggest further that what substances and structures are present in any single cell may in many respects be dependent upon the precise location of that cell in the functioning gross anatomical structure of which it is part.

N. K. W.

BIOPHYSICS SEMINAR

February 2, 1959

VIRUS INDUCED ACQUISITION OF METABOLIC FUNCTION. By Seymour S. Cohen, University of Pennsylvania, Philadelphia.

When certain of the T-series bacteriophages infect susceptible forms, such as *Escherichia coli,* profound alterations in bacterial cell metabolism occur. Thus, in the normal cell, of every four phosphorus atoms taken up,

one is incorporated in DNA while three go into RNA; in the infected bacterium, on the other hand, all four atoms become associated with DNA molecules.

Recent work on the enzymic properties of *E. coli* indicate in part, how such drastic changes in metabolism may occur. Following injection of the genetic material of T-even phages into *E. coli*, two new enzymes, 5-hydroxymethylase and thymidylate synthetase, appear in cell homogenates. The former is active in the final biosynthetic step in the formation of 5-hydroxymethylcytosine. This compound is one of the four bases found in phage DNA; it is never present in bacterial DNA. The second enzyme was found when a thymine deficient bacterial mutant (that could not synthesize DNA) was infected by $T_{2\text{-}B}$ phage. Phage DNA was synthesized. The enzyme that bridged the missing link in the chain of thymine synthesis was formed in great quantity after infection.

It is noteworthy that neither of the inducible enzymes appear following infection by most T-odd phages, by $K_{12}\lambda$ phages, or by "ghosts" of T2 phages. The only known exception is T5, a form lacking 5-hydroxymethycytosine. Just as would be expected, *E. coli* infected with this virus contain thymidylate synthetase but never any 5-hydroxymethylase. Since such phage-induced metabolic properties are so closely related to the requirements of phage reproduction, it is thought that relatively simple systems like this one may be an excellent place to study the action of genetic material. One proviso is, however, that a specialized system is being dealt with—one concerned almost completely with DNA production and metabolism.

N. K. W.

TUMORS AND TUMOROUS CONDITIONS OF THE BONES AND JOINTS. By Henry L. Jaffe. Philadelphia, Lea & Febiger, 1958. 629 pp. $18.50.

Much of the value of a text dealing with a special branch of pathology lies in its ability to correlate morbid anatomy with clinical observations. Dr. Jaffe, who has been pathologist to the Hospital for Joint Diseases in New York for three decades, is a master at explaining the facts and speculations of bone pathology to orthopedic surgeons and is equally adroit at rationalizing the clinical exigencies which govern the study, diagnosis, and management of bone tumors to his fellow pathologists. Primary neoplasms of bone are not common fare in the laboratory. By virtue of his position, it has fallen to Dr. Jaffe's lot to be responsible for the care, preservation, and study of an uncommonly large number of such specimens, many of which are of the sort that one encounters once in a lifetime. Employing a rational and empirical approach, the author has acquitted himself nobly of the task of epitomizing the known factual material about bone tumors and related lesions. Over and above this goal, he achieves a lucid synthesis of clinical, radiological, and pathological features of each neoplastic entity considered. It is much to his credit that he has been able also to assimilate and evaluate for his readers the large volume of theoretical material which has been published in this field and will probably dominate the field for many years.

The text is ably supported by almost 200 full-page plates, each containing several separate illustrations. The illustrative material includes x-rays, gross specimens, and well chosen microscopic fields. In many instances gross specimens are presented in juxtaposition to the x-ray of the lesion in question, and one is privileged to see not only the external surface but the cut surface in the radiological plane. All the plates are arranged recto, the legends being verso; space for illustration is therefore not sacrificed to legends, and the legends are extremely detailed. It is practically impossible to look at the pictures without understanding what is going on. The selection of microscopic fields and the cropping of prints is always a painful and time-consuming business. The author's efforts have not been wasted; there are no pictures with blurred margins, no evidence of faulty histological technique, poor contrast, awkward composition, etc. The plates are a delight to the microscopist's eye. The same high standard of selection applies to the roentgenograms.

For the reader already familiar with the literature on bone tumors there are many fascinating passages. Among these is the large stride in classification in which Jaffe goes far beyond Phemister's schema, currently in vogue in many circles. There is the blunt statement that Willis's doubts regarding the existence of such an entity as Ewing's tumor have turned out to be unjustified, a statement as dogmatic as those of the British master and based on more evidence. In one short paragraph Jaffe puts Albright's disease (fibrous dysplasia of bone associated with precocious puberty, abnormal pigmentation, etc.) into perspective: ". . . for every case of the florid (Albright) type there are about 30 or 40 cases . . . in which the fibrodysplastic involvement of the skeleton is limited . . . and is not associated with any striking cutaneous pigmentation, apparently not with skeletal precocity, and certainly not with sexual precocity. In spite of their small number, these

florid cases bulk large in the literature, because their spectacular qualities make them attractive subjects for reporting." Equally commendable is the discussion of the nature and genesis of adamantinoma of the limb bones, a highly controversial topic; Jaffe presents all current theories fairly and deflates them all equitably.

In an age so conscious of radiation and its effects upon tissues, the reviewer cannot resist singling out for mention the chapter on radiation injury and postradiation sarcoma of bones. The text is balanced and sane, leaving plenty of room for filling out the framework with new data when such becomes available. The illustrations of radiation osteitis are "classical"—in the sense that the classical picture is the best illustration as opposed to what one usually gets to see.

One of the features that will appeal to both clinicians and pathologists alike is the section on differential diagnosis which accompanies the text dealing with specific lesions. The paragraphs are not long, but they are the distillate of much practical experience and good medical judgment.

The bibliographies following each chapter are well selected and will guide the reader to both classical descriptions of lesions and good contemporary reviews and significant case reports. One misses, perhaps, the reference to Antoni's original description of type A and type B tissue in neurilemmonas, but this is a minor point. There is certainly no effort to avoid relevant references merely because they are in a foreign tongue.

Each reviewer is entitled to cavil at a certain point. One queries whether the chondromyxoid fibroma and the desmoplastic fibroma are quite so strictly separable entities as Jaffe states. Could they not be different phases of a common process? Are they completely unrelated to the non-ossifying fibroma? Speculation outruns data at this point. One also can speculate with the author regarding the route taken by the metastases from the giant-cell tumor of the upper end of the femur appearing 2½ years later in the prepuce (See Fig. 1 B).

We can only hope, in conclusion, that Dr. Jaffe, having dealt with neoplastic bone disease so successfully, will now devote his efforts to summarizing his experiences with metabolic and degenerative diseases of bone.

WILLIAM B. OBER

BONE AND RADIOSTRONTIUM. By Arne Engstrom, Rolf Bjornerstedt, Carl-Johan Clemedson, and Arne Nelson. New York, John Wiley & Sons, Inc.; Stockholm, Almqvist & Wiksell, 1957. 139 pp. $8.75.

The current interest in hazards of nuclear bomb testing and possible induction of bone tumors by radioactive strontium makes this volume most timely. It presents the results of experimental work in metabolism of strontium and discusses certain physical aspects of the problem. Investigative techniques are presented in some detail.

The preliminary section of the book deals with a summary of data found after nuclear explosions as well as facts gathered in animal experimentation on modes of absorption, excretion, and toxicity of radiostrontium. Note is made of the biological discrimination against strontium in favor of calcium

which occurs in nature in the several steps of the food chain from plants to man. This section is followed by a comprehensive bibliography of American and European references on the subject, including some Russian authors.

A presentation of the authors' own experimental methods in determining the distribution of radiostrontium in bone then follows. The techniques which have been utilized include differential x-ray absorption in microradiography and microinterferometry. The nature of the bone salt and effect of strontium replacement of calcium in the crystal was studied by x-ray diffraction. It is concluded that the introduction of strontium into the living skeleton most likely results in its adsorption onto the surface of the apatite crystals.

Localization of strontium-90 in bone was carried out also by means of autoradiography and the time relationship of distribution carefully elucidated. It is of interest to note the marked inhomogeneity in distribution with marked concentrations in areas of rapidly growing and poorly mineralized bone.

Calculation of radiation dose from the radioisotope was carried out using a theoretical sandwich model which seemed justified by the above investigations into the distribution in bone. Account is taken of the possibility of marked local concentrations in placing a suggested value for the maximum permissable body burden of strontium-90 as 0.1 microcurie.

The presentation is both precise and thorough. Numerous illustrations and charts supplement the text. The book is invaluable as a reference for experimental methods and systems of dosage calculation for those doing investigative work in this field.

HAROLD L. ATKINS

THE CHEMICAL DYNAMICS OF BONE MINERAL. By William F. Neuman and Margaret W. Neuman. Chicago, University of Chicago Press, 1958. 209 pp. $5.00.

This book serves two useful purposes. First it is an excellent review of bone physiology including both the data and theories that form the basis of our current ideas in this area. Second, it demonstrates the need to integrate the basic physical sciences with physiology and thus apply the ideas and methods of these disciplines to the many problems that remain unsolved.

The book is divided into two main sections. The initial portion is devoted to a review of basic physical science as it pertains to bone. Thus such topics as solution chemistry, solubility theory, activity coefficients of ions, ion exchange, and crystallography are discussed. This includes both *in vitro* and *in vivo* data, pointing out many of the limitations and differences implicit in *in vivo* observations.

After developing these basic concepts the authors go on to the physiology of bone, dealing mainly with the effect of parathormone and vitamin D, as well as the mechanism of bone calcification. They develop the interesting and provocative theory that the citrate ion plays a major role in the dynamic equilibrium between bone deposition and bone reabsorption. They further suggest that these alterations in the citrate ion occur as a result of the effect of parathyroid hormone and vitamin D on the metabolism of bone cells.

Although the hypothesis presented has much experimental data to support it, the authors are quick to point out the limits of our present knowledge and indicate the areas where further investigation is needed. In their own words the ideas offered are at least tentative if not temporary. Whether or not the theories set forth will ultimately be proven correct is less important, for the reader comes away with a greater knowledge and understanding of bone physiology.

This monograph is a must for research workers in the field of bone and electrolyte metabolism. The sections on the physical sciences may be less useful to the clinician, but those who are interested in keeping abreast of the times will find it quite valuable.

HOWARD LEVITIN

MOMENTS OF DISCOVERY. Edited by George Schwartz and Philip W. Bishop, with an Introduction by Linus Pauling. New York, Basic Books, 1958. 2 vols. xvii+xi+1005 pp.; over 50 illustrations. $15.00.

The anthologist must of necessity have a certain courage, for he undertakes to select those writings which seem to him most representative, most interesting, most important. To decide upon the best works of a poet or a literary period is difficult enough; to define successfully and to the satisfaction of all readers the high points in the written record of all science is nearly impossible. The editors of *Moments of Discovery*, then, set themselves a large task.

Selections from the writings of nearly ninety individuals appear in these two volumes. Beginning with Hippocrates, the great men of science, and some not so familiar, set forth their observations and ideas. The major fields are well represented, both in variety and in historical perspective. The last and latest quotation is from J. Robert Oppenheimer. One cannot complain seriously about the list of the men and the woman (Marie Curie) chosen to represent scientific thought.

But *what* was selected is another matter. "True creativity," says the Preface, ". . . is revealed by the ability to crystallize meaningful concepts out of observations which at first glance are chaotic and patternless." This ability to synthesize is a hallmark of the productive scientist, and the circumstances wherein the synthesis is achieved, often in a flash of intuition, the actual "Moment of Discovery," are an exciting problem for study. This reviewer can never read without awe and exaltation William Harvey's simple description of how he first recognized the circulation of the blood—". . . I began to think whether there might not be a MOTION, AS IT WERE, IN A CIRCLE. Now this I afterwards found to be true . . ." This passage appears in Volume Two, as does Theobald Smith's great paper on Texas cattle fever, Pasteur's on the treatment of rabies, and Harrison's observation of the growth of the neuron and invention of tissue culture. Ben Franklin's sprightly letter on "The Kite Experiment" tells clearly how he captured the "electric fire." But too often the pages selected describe, not The Moment, with its realization of discovery shining from scrawled laboratory notes, but instead the great contribution as seen in the retrospective

and solemn evaluation of later weeks or years. For men like Linnaeus and Darwin, whose conclusions seem to have been reached only gradually and laboriously, perhaps there was no Moment of Discovery; certainly the passages from their writings are pedestrian.

Particularly interesting is a section on "Technology before Science," with quotations from George Sarton and V. Gordon Childe. Here is an all too brief treatment of the technology that came before science, for example the "improvisations of a farmer remote from industrial facilities. . . . An interesting example of this is found in the solutions to the problem of how to keep a gate closed in a field fence. There are many ingenious and effective designs to be seen that have rarely been repeated and certainly never patented" (page 102).

Each passage by a scientist is introduced by the editors' historical and biographical sketch, the sketches in turn being grouped and discussed in terms of broad categories. The background material is interesting but sometimes diffuse. A number of illustrations from original works are reproduced, not always well. Although the book in general is remarkably free of typographical errors, the dates given for the birth of Robert Hooke (page 387) and Pierre Curie (page 888) and for the death of Francesco Redi (page 405) and William Harvey (page 575) do not agree with those cited by other authorities. There is a rather brief index.

For the authors' aim for this book there can be only admiration; one feels much disappointment that the product of twenty years of work is so uneven.

THOMAS R. FORBES

ATRIAL ARRHYTHMIAS, DIGITALIS AND POTASSIUM. By Bernard Lown, and Harold D. Levine. New York, Landsberger Medical Books, Inc., 1958. 222 pp. $6.90.

This little monograph is a most exhaustive and well-documented account of the effects of digitalis on the atrium with particular (in fact almost exclusive) relation to paroxysmal auricular tachycardia with A-V block. The evidence for the relationship of this arrhythmia to digitalis intoxication, either due to excessive doses of digitalis or to deficient cellular potassium, is presented in great detail. Both the clinical features and electrocardiographic distinctions of this arrhythmia are well discussed. Problems of differential diagnosis and therapy are ably covered. There is an interesting section at the end of the book on the theoretical basis for this particular manifestation of digitalis intoxication involving the contrasting vagotonic and direct myocardial effects of digitalis as well as the relationships of acetylcholine and potassium to digitalis action.

This text is valuable particularly for its emphasis on paroxysmal auricular tachycardia with A-V block as a special manifestation of digitalis intoxication. It is designed particularly for the cardiologist and electrocardiographer but would certainly be of considerable interest to internists in general who are particularly concerned with the cardiovascular field.

ALLAN V. N. GOODYER

PRINCIPLES OF GENETICS. 5th ed. By Edmund W. Sinnott, L. C. Dunn, and Theodosius Dobzhansky. New York, McGraw-Hill, 1958. 459 pp. $6.75.

This thorough revision of a standard text is most welcome in a field which has witnessed so many recent changes. As stated in the preface, the purpose has been ". . . to keep the primary emphasis on elementary principles but at the same time to make the student aware of the wide range of problems disclosed by recent research." This admirable intent has met with varying degrees of success.

The re-organization of the introductory chapters and those dealing with classical genetics has resulted in a more interesting presentation of the material. Many of the clumsy, dull phrases of older editions have been removed or rewritten in a more lucid style. The outstanding feature of the book is still the historical approach to a subject which, during its development, has contributed so much to scientific thought.

A major alteration is the incorporation of the material covering the statistical approach to genetics into a single, concise chapter at the close of the book. This chapter, by Howard Levene, is well developed and self-contained. Unfortunately, if read at the time suggested in the text, the student may be confused by references to genetic principles not yet covered in his reading. The problem of integrating this chapter with the main part of the text is clearly left to the lecturer.

It is not surprising to find that a large proportion of the new material involves the recent studies on microorganisms, their reproduction and genetic significance. This has been balanced by the inclusion of more material on human heredity and the methods used in its analysis. An increased emphasis on biochemical aspects is apparent throughout.

Several new chapters have been added, of which the one on "Crossing, Selfing, Inbreeding, and Heterosis" is especially well done. Pseudoalleles and position effects, although including some new material, did not fare so well. The chapters dealing with physiological and developmental genetics and the role of the cytoplasm in heredity and development are particularly disappointing in being largely a collection of isolated studies with little attempt at pulling them together into a meaningful whole or confronting some of the basic problems involved. This may be a reflection of the surprising avoidance of Goldschidt's contributions to the thinking in these fields. Such restraint in presentation might have been more applicable to the discussion of DNA in "The Elements of the Genetic System." Most distressing was the misrepresentation of the work of Fraenkel-Conrat (p. 375).

The new format is appealing and the diagrams, entirely redone, are especially clear and dynamic. Many new problems have been included and the bibliography, accompanied by suitable annotations, has been restricted to the end of the book. The latter is indeed an improvement, but it is regrettable that journal references have been deleted.

In spite of the various criticisms leveled against it, this edition, when taken as a whole, represents an improvement over the previous one. It is still probably the best introductory text to the science of genetics.

W. W. DOANE

THE TEACHING AND LEARNING OF PSYCHOTHERAPY. By Rudolf Ekstein and Robert S. Wallerstein. New York, Basic Books, Inc., 1958. 334 pp. $6.50.

These authors have written the first book on the process of supervision in the field of psychotherapy. This publication is the outgrowth of pioneer work done in a carefully organized manner over the past ten years at the Menninger Foundation. At the outset, the authors make the major point that ". . . we do not teach psychotherapy as a series of specific interactions, but how to carry on psychotherapeutic activity." Though in fact they have written primarily for those students and supervisors of psychotherapy who work in the framework of psychoanalytic principles, their book is oriented in such a way that it contains much of value for anyone engaged in the learning and teaching of psychotherapy.

At the outset, they view the institutional setting in which early clinical teaching takes place and make thoughtful references to the complexity of the professional society in which the beginning student of psychotherapy finds himself. They depict this as "The Clinical Rhombus" with hospital administrator, psychotherapy supervisor, the student of psychotherapy, and the patient at the apices of this geometrical figure with numerous paths of interaction available.

The major section of the book is devoted to the development of a dynamic understanding of the parallel processes of treatment and supervision. The authors report selected supervisory and treatment hours in sufficient detail for the reader to understand the unfolding and development of the essential themes. They successfully deal with issues and dilemmas of how supervision is related to psychotherapy and remind us that in both we are dealing with affect-laden relationships in which a wide range of human emotion may be revealed and explored. They make a point of suggesting that the supervisor not see his student as a collection of startling psychopathology but as a person in a learning situation who makes use of his characteristic patterns of coming to grips with new and sometimes very threatening experiences.

The supervisory methods described here make much use of written interview material turned in to the supervisor for his study prior to the supervisory sessions. Though one may question the value of this particular routine, this procedure does not imply a static or didactic teaching situation. Rather, these notes are used as a springboard for discussion of treatment and supervision. In the closing section, they discuss the evaluation of supervision by student and supervisor and introduce questions related to the entire problem of evaluating a student's progress in learning and the selection of students who may have promise as psychotherapists.

A very excellent and comprehensive "Guide to the Literature" is appended. This volume should be read by all students and teachers who have serious interest in the learning and teaching of psychotherapy.

It should provide many ideas for other workers to study teaching and learning in a wide variety of clinical settings.

EDWIN C. WOOD

THEORY OF PSYCHOANALYTIC TECHNIQUE. By Karl Menninger. New York, Basic Books, Inc., 1958. 206 pp. $4.75.

A contribution to theoretical thinking about psychoanalytic technique by a leading psychoanalyst such as Karl Menninger is noteworthy. From this point of view alone, this book commends itself to anyone utilizing psychoanalytic principles and practices in his work, anyone interested in the philosophical and practical impact of psychoanalysis on society today, or one whose contact with psychoanalysis may have been only from the vantage point of the couch. In addition, Dr. Menninger brings to this highly complex subject his characteristically clear, concise style devoid of oversimplification. In fact, by organizing the material to follow the temporal sequence of psychoanalytic treatment, from initial patient-doctor contact to termination, much of the crisis and suspense inherent in the experience itself is conveyed and the book's readability enhanced.

Dr. Menninger conceptualizes the psychoanalytic "contract" as a special form of two-party contract in our society. Starting from the model of the contract between grocer and customer where money is exchanged for a product, he progresses through contracts based on the performance of a service to the specialized service contract of a "qualified, authorized, prepared, willing psychoanalyst" who "suspends judgment and interprets" in relation to a "sick patient" who "cooperates, communicates self observation and pays [money]" (Fig. 5, page 28). The ensuing process experienced by the patient in the working out of this contract is described by Dr. Menninger as a psychic regression. This regression is stimulated by factors in the treatment situation, not the least of which is the parsimony of intervention on the part of the analyst. It is precisely the reversal of this process of regression and the patient's finding of new and more adequate goals that constitutes the healing portion of the psychoanalysis. Dr. Menninger indicates that this reversal is partly aided by the analyst's interpretations and other interventions but unfortunately does not attempt a real theoretical formulation of why the regression does in fact undergo reversal.

This concept of the analytic process as a regression is distinct from the more classical formulation of the process as primarily the development of transference neurosis and the resolution of this transference neurosis; the author makes an attempt to clarify the distinction. In addition, he discusses such basic phenomena as patient resistance, analyst countertransference, and the nature of interpretation. There are also interesting discussions throughout the book of practical technical issues such as the rationale for charging a fee, criteria for terminating the contract, and the appropriate timing and types of interpretation used by the analyst.

This book, the first from this author in several years, is thus of interest to the general reader but virtually a must for the serious student of psychiatry.

ALBERT ROTHENBERG

UROLOGY IN GENERAL PRACTICE. By Frank C. Hamm and Sidney R. Weinberg. Philadelphia, J. B. Lippincott Co., 1958. 293 pp. $6.00.

In this excellent small volume, the authors aimed to "Introduce the medical student and resident physician to the field of Urology" and "to consider the

. . . problems against the larger spectrum of general medicine." They have done this with almost the brevity of an abstract, yet with the clarity of a fuller presentation. The ample illustrations are well chosen and cleanly executed. Good bibliographies are supplied. The volume represents good current urological thinking and may be recommended as a manual for students and a reference for practitioners.

B. MARVIN HARVARD

WATER AND ELECTROLYTE METABOLISM IN RELATION TO AGE AND SEX. Vol. 4 of the Ciba Foundation Colloquia on Aging. G. E. W. Wolstenholme and Maeve O'Connor, Eds. Boston, Little, Brown and Co., 1958. 327 pp. $8.50.

This volume is the transcript of a series of conferences held in January, 1958, in London and presided over by R. A. McCance whose own contributions to the developmental aspects of renal physiology are classic. The list of contributors is impressive and includes outstanding workers in the field of "the inorganic chemistry of the body" from many countries—Borst, Adolph, Wallace, Davson, Kerpel-Fronius, Hingerty, Desaulles—these are only a sampling of the participants.

One picks up this volume eagerly to listen in on the informal responses of great men to stimulating subjects. Perhaps partial disappointment is inevitable. As always with published symposia, much of the discussion is disjointed and arguments are often left hanging in midair and not pushed to their logical conclusion. Some papers represent material that is not new; others are trivial or preliminary. But, as always, with the Ciba Symposia, there are bright spots which make the entire volume worthwhile. Dr. Hugh Davson's chapter on "Cellular aspects of the electrolytes and water in body fluids" is a model of clarity. Dr. Nathan Shock has an excellent summary of his work on the role of the kidney in electrolyte and water regulation in the aged. Fourman has a stimulating paper dealing with hypernatremia and hyponatremia with special reference to cerebral disturbances. Other chapters include, "The development of physiological regulation of water content," by E. F. Adolph, "Hormonal aspects of water and electrolyte metabolism in relation to age and sex," by G. I. M. Swyer, "Clinical consequences of the water and electrolyte metabolism peculiar to infancy," by E. Kerpel-Fronius, and "The development of acid-base control," by E. Widdowson and R. A. McCance, as well as many others.

This book is recommended to physiologists, pediatricians, and internists who are interested in the climate of current thought about the physiology of water and electrolytes.

FRANK EPSTEIN

POLYSACCHARIDES IN BIOLOGY. Transactions of the Second Conference. Georg F. Springer, Ed. New York, Josiah Macy, Jr. Foundation, 1957. 245 pp. $5.00.

This publication contains the transactions of the second multiprofessional conference on polysaccharides in biology sponsored by the Josiah Macy, Jr. Foundation which was held in April 1956. The subject of the conference is

divided into three related categories: sialic acid and related compounds; interaction of polysaccharides and viruses; and pyrogens. The conference itself was limited to twenty-five participants from various disciplines in the medical sciences.

The editor has succeeded in preserving the informal nature of the meetings by presenting the entire conference in the form of the original discussion complete with interruptions and questions. While the publication of these exchanges furnishes the reader with an interesting glimpse of the personalities and prejudices of the participants, the subjects under discussion are consequently presented in a somewhat disorganized sequence with many seemingly irrelevant interruptions. On the other hand, many important questions are injected which might otherwise go unanswered for the reader who is not completely familiar with the area under discussion.

This reviewer is left with the impression that the conference succeeded admirably with its announced purpose of ". . . promotion of communication, the exchange of ideas, and the stimulation of creativity among the participants." However, the lapse in time between the conference and the publication has removed much of the value of the publication as far as sharing the conference with a larger audience. Thus, most of the discussion concerned with sialic acid and related compounds has little significance as a result of subsequent advances in the determination of the structure of these compounds. Although suffering from the publication time lag, the other discussions do provide informative reviews of various areas of research which apparently involve polysaccharides or polysaccharide complexes, e.g., the interaction of influenza virus and other viruses with erythrocytes; the properdin system; the pyrogenic activity of bacterial endotoxins.

The nature of the published transactions make them of little value to the student as a reference source for his bookshelf. However, in spite of the material being two years old, the lively discussion, questions, and answers will provide an evening of interesting and informative reading.

JOHN A. DE MOSS

SURGICAL CONVALESCENCE. Annals of the New York Academy of Sciences, vol. 73, Art. 2, pp. 381-538. F. Curtis Dohan, *et al.*

Surgical Convalescence is the product of a symposium held jointly by the New York Academy of Sciences and the American College of Surgeons. It is an attempt to present some of the salient features of recovery from a surgical illness in a series of brief essays. This group of papers is especially interesting in that it covers many aspects of convalescence which are not usually dealt with by physicians or surgeons.

Among the articles devoted to the physiological aspects of recovery, Dr. Francis D. Moore discusses evaluation of the trauma and the phases of convalescence in the man who survives a serious physical insult. He gives special attention to the role of nitrogen metabolism, pointing out that the major source of protein for catabolism following injury, and therefore the beneficiary of positive nitrogen balance during recovery, is skeletal muscle. As a corollary to this observation he notes that physical rehabilitation must

parallel, but not exceed, caloric intake if adequate convalescence is to take place.

An excellent chapter on the endocrine, electrolyte, and water metabolism of the post-traumatic patient is contributed by Dr. James D. Hardy. He elaborates his thesis of the totality of body involvement in response to surgical trauma in a factual manner, but without allowing a plethora of data to obscure his argument. Dr. J. Engelbert Dunphy presents a speculative review of tissue repair at the site of injury.

In addition to the experimental papers there are a number of statistical reports, notably those by Drs. Allen and Martin and Drs. Moss and Curtis, attempting to evaluate the duration of surgical convalescence.

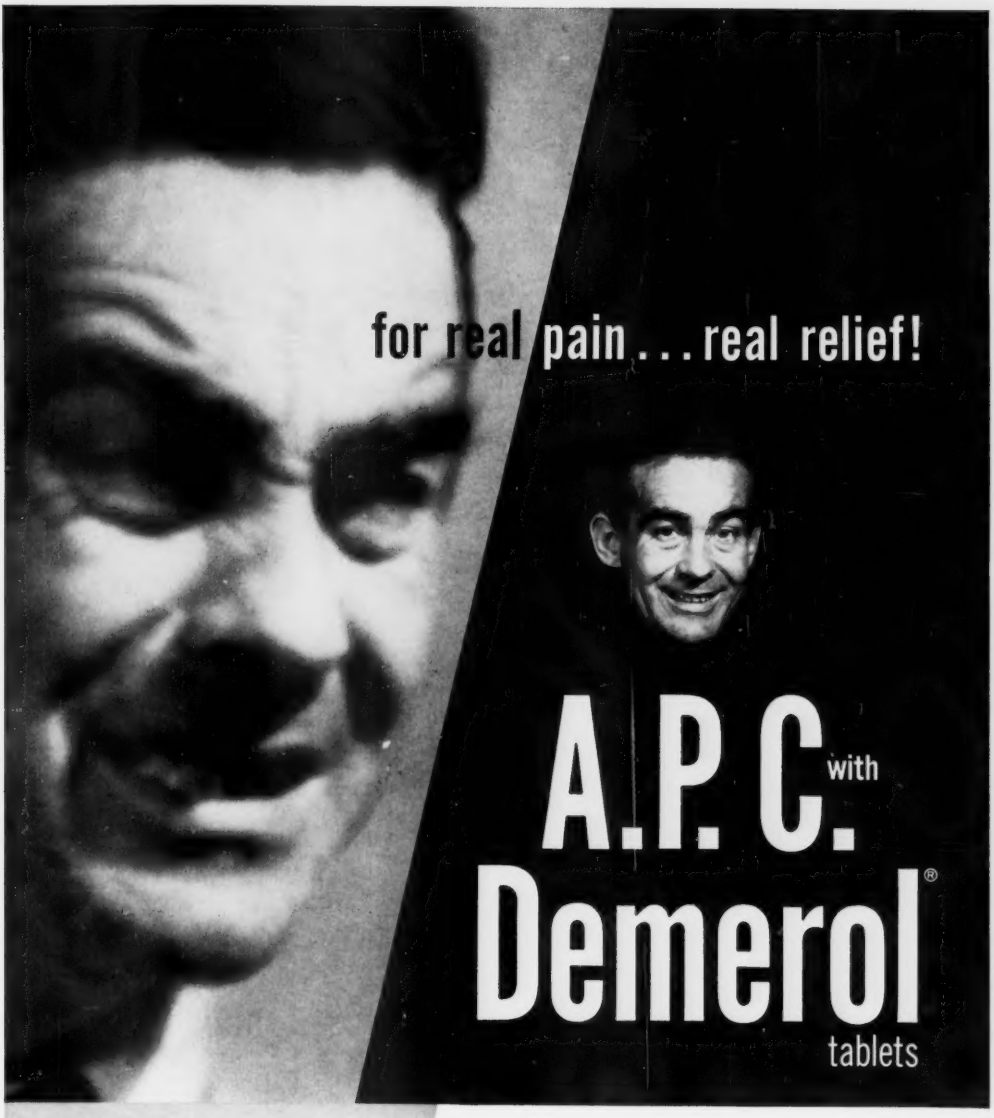
Dr. Howard Rusk discusses the use of rehabilitative measures in promoting recovery, and Drs. Henry Taylor and Ancel Keys present a study designed to elaborate criteria for establishing "fitness" following recovery from injury.

There are also several articles concerned with the tremendous sociological importance of absenteeism secondary to surgical illness. Dr. Paul Hardy reports the astonishing fact that in a single year eight million patients underwent surgical procedures with a loss to the national economy of over four billion dollars.

Finally, Dr. Arthur Sutherland discusses a problem which is often mentioned, but all too frequently poorly appreciated by those who do the surgery, namely, the influence which psychological factors have upon the patient's recovery and return to health.

In conclusion, this little collection of essays attempts to present an overall picture of the convalescence of the surgical patient. It is highly successful, as far as it goes, in illuminating a large number of different facets of a complex problem. If it has its failures, they lie in the inability of a scientific round table to present a complete picture of either the individual or the group and their response to disease. For this kind of unity great artists such as the creators of the *Magic Mountain* or *The Plague* are necessary, and most people would say that it is far beyond the scope of the New York Academy of Sciences to invite a Thomas Mann, were he alive, or a Camus to their discussions. Unfortunately, they are probably right.

JOHN H. SIEGEL



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